ENGINEERING TISSUE CONSTRUCTS TO MIMIC NATIVE AORTIC AND PULMONARY
VALVE LEAFLETS’ STRUCTURES AND MECHANICS

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ABSTRACT

There are several disadvantages correlated with current heart valve replacement, including anticoagulation therapy for patients with mechanical valves and the low durability of bioprosthetic valves. The non-viable nature of such devices is a critical drawback especially for pediatric cases due to the inability of the graft to grow in vivo with the patients. A tissue engineered heart valve (TEHV) with remodeling and growth ability, is conceptually appealing to use in the surgical repair and could serve as a permanent replacements when operating for pediatric valvular lesions. It is critical that scaffolds for functional heart valve tissue engineering, be capable of mimicking the native leaflet’s structure and mechanical properties at the time of implantation. Meanwhile, the scaffolds should be able to support cellular proliferation and native-like tissue formation as the TEHV remolds toward a scaffold-free state.

Our overall hypothesis is that an “ideal” engineered construct, designed based on native leaflet’s structure and mechanics, will complement a native heart valve leaflet in providing benchmarks for use in the design of clinically-applicable TEHV. This hypothesis was addressed through several experiments conducted in the present study. To establish a functional biomimetic TEHV, we developed scaffolds capable of matching the anisotropic stiffness of native leaflet while promoting native-like cell and collagen content and supporting the ECM generation. Scaffolds with various polymer contents (e.g., poly (glycerol sebacate) (PGS) and poly (ε-caprolactone) (PCL)) and structural designs (e.g., microfabricated and microfibrous scaffolds), were fabricated based on native leaflet’s structure and mechanics. It was found that the tri-layered scaffold, designed with assembly of microfabricated PGS and microfibrous PGS/PCL was a functional leaflet capable of promoting tissue formation. Furthermore, to investigate the effect of cyclic stress and flexure individually on the TEHV development, we designed a simple and novel stretch-flexure bioreactor in which samples were subjected to well-defined stimulations with a controlled strain-rate. The stretch and flexure was found to accelerate and increase tissue formation on the microfabricated PGS scaffolds cultivated in the bioreactors.
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PREFACE

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ACRONYMS

$\alpha$-SMA; alpha smooth muscle actin
SMC; smooth muscle cell
EC; endothelial cell
VIC; valvular interstitial cell
VEC; valvular endothelial cell
BMSC; bone marrow-derived mesenchyme stem cell
RVE; representative volume element
FE; finite element analysis
RC; radius of curvature
PD; preferred fiber direction
XD; cross-preferred fiber direction
PGS; poly (glycerol sebacate)
PCL; poly (caprolactant)
P4HB; poly(4-hydroxybutyrate)
DMEM; Dulbecco's Modified Eagle Medium
HBSS; Hank’s Balanced Salt Solution
PBS; fetal bovine serum
UTS; ultimate tensile strength
PA; Pulmonary artery
PV; Pulmonary valve
AV; Aortic valve
NOMENCLATURE

$\varepsilon_f$; strain to failure
$v$; volume fraction
$\alpha$; diamond pores angle, gear rotation angle
$\theta$; half of the central angle of the circular arc
$Y_\sigma$; Yield stress
$Y_\varepsilon$; Yield Strain
$E$; specimen effective stiffness
$E_f$; fiber longitudinal stiffness
$\rho$; microfabricated scaffold density
$R_c$; Radius of curvature
$S$; Scaffolds length in the flexure condition (Arch length)
1 INTRODUCTION

1.1 HEART VALVE ANATOMY AND STRUCTURE

The heart contains four chambers connected by valves in human and mammalian animals (Fig. 1). Heart valves serve to control blood flow through the heart when heart ventricles pump the blood to the lungs (deoxygenated blood flows through the right ventricle) and the systemic circulation provide the oxygen and nutrition for the organs (oxygenated blood flows through the left ventricle) [1].

Figure 1: Gross anatomy of the four-chamber mammalian heart. Image adapted from Edwards Lifesciences, Irvine, CA (www.edwards.com/PatientsAndFamilies/MyHeart/Anatomy/).

1.1.1 Semilunar Valves Organ Level Structure

The aortic and pulmonary valves with a unique tri-leaflet structure of semilunar (i.e., half-moon) shape (Fig. 2) are similar in the structure and physical characteristic. These valves consist of three valve leaflets with the surrounding aortic or pulmonary root. The valve is separated on the ventricular side by a semilunar line of attachment named the annulus fibrosis, and on the aortic or pulmonary side by a circular
ridge of thickened tissue restricting the apices (i.e., commissures) of the semilunar leaflets referred to as the sinotubular junction [2]. The leaflet body extends from the annulus fibrosis to the free edge, creating a pocket-like cavity between the leaflet and its associated aortic or pulmonary root wall. During valve opening and closing, these three voids fill with blood and ultimately press together which the phenomena is known as leaflet coaptation. The leaflets meet at the coapting surfaces or lunulas. Nodulus Arantii is a small nodule of thickened tissue located at the center of each lunula which reduces regurgitation. The circumferential angular distance from commissure to commissure is approximately 120 degrees considering the fact that leaflets are similar sizes. The triangular regions of aortic root known as interleaflet triangles which are descending from each of the three commissures and demarcated on either side by the semilunar lines of the annulus fibrosis [2].

Figure 2: Surgical anatomy of a semilunar heart valve. Image (A) from Thubrikar [3], (B) is an open aortic root of fresh porcine heart for dissecting valve leaflets. (C-D) show the semilunar structure of heart valve leaflets obtained from [4].
1.1.2 Tissue Structure of the Aortic and Pulmonary Valves

The tissue-level microstructure of the semilunar valve leaflet is based on heart valve functionality to withstand the cyclic loading of the cardiac function (Fig. 3). Here, we describe the aortic tissue level structure specifically since both aortic and pulmonary leaflets include similar structure. The human aortic valve leaflet thickness is ~ 700 microns at the annulus fibrosis to about 200 microns at the free edge. Valves leaflets are a bit thinner in the other species as measured in the following study. Over the thickness, the leaflets are a complicated 3D architecture with a tri-layered structure. The layer facing the aortic root is known as the zona fibrosa (aortic side), the soft middle layer is zona spongiosa (central), and zona ventricularis (ventricle side) is the surface facing the ventricles. The fibrosa is a fibrous layer consists primarily of circumferentially-oriented type I collagen fibers. In addition to the fiber level crimp intrinsic to type I collagen, the fibrosa also exhibits a macroscopic corrugated structure which is presumed to allow for radial leaflet expansion. On the opposite side of the leaflet, the ventricularis layer consists primarily of elastin, with some loosely arranged type I collagen fibers. These fibrous layers provide the required strength to withstand the transvalvular pressures during valve closure (diastole). The ventricularis is thought to be important both in stabilizing the corrugated collagen structure of the fibrosa, and to provide a tensile pre-load in the leaflet [5]. The spongiosa layer is situated between the fibrosa and ventricularis and is a highly hydrated component, which consists primarily of water, proteoglycans, and glycosaminoglycans (GAG). GAG are long, negatively-charged polysaccharides that serve to preserve water within the spongiosa, both in their free form, as well as when bound to a protein core (i.e., proteoglycan) [6]. This layer exists to reduce shear stresses and buckling during leaflet bending and are attributed to the leaflet durability [7, 8].
Figure 3: Valve leaflets comprise of three interconnected layers. AV cross sections (circumferential and radial). The fibrosa (F) is comprised primarily of circumferentially oriented collagen (yellow). The spongiosa (S) is a gelatinous layer containing GAGs and interstitial cells. The ventricularis (V) contains elastin[9].

1.1.2.1 Collagen orientation in native heart valve tissue

The mechanical properties and associated function of heart valve leaflets depend intimately on collagen composition and orientation [9-11]. Thus, in a preliminary study we attempted to image the collagen fibers in a native porcine (pulmonary and aortic) heart valve leaflets (Fig.4). Collagen were imaged by confocal reflectance microscopy technique, using an excitation wavelength of 500 nm as recently used in our lab for imaging collagen gelled within the pores of accordion-like honeycomb PGS scaffolds and native porcine pulmonary heart valve leaflets (Fig.4 C-D). The results showed that the
fibrosa layer is predominantly aligned packed collagen fibers. In addition, native valve leaflets typically exhibit well-defined, highly autofluorescent collagen bundles (Fig.5) that are packed densely and three dimensionally organized within the tissue layers. The valve leaflets endure large cyclic deformations (changes as high as 50%) and the pressure differences rising during the cardiac cycle [12]. When the valve is closed, the collagen fibers are fully unfolded, so the load-bearing element shifts from elastin to collagen and stress rises. During systole, the elastin fibers restore the contracted configuration of the cusps[13].

Figure 4: Confocal reflectance microscopy of collagen in PGS scaffolds and native pulmonary valve tissues. (A) Native pulmonary leaflet (B,D) DIC pictures of the two zones on the leaflet ventricularis surface and (C,E) corresponding confocal image of those points.
1.1.3 Cellular Arrangement of the Native Valve Leaflet Tissue

The aortic and pulmonary valve leaflets are populated by different cell types generally referred to as valvular interstitial cells (VICs). They mostly exist in the spongeosa layer and at the blood-leaflet interface (on the surfaces of fibrosa and ventricularis), the leaflets are covered with a thin confluent layer of valvular endothelial cells (VEC). VICs phenotype is similar to smooth muscle cells (SMC) and fibroblasts. Also, due to expression of both fibroblast markers such as vimentin, SMC markers and alpha smooth muscle actin (α-SMA), they have been characterized as VA-type myofibroblasts [14-16]. Healthy adult VICs exhibit a quiescent, fibroblast-like phenotype, expressing vimentin and, to a lesser degree, αSMA (myofibroblast marker) [17]. During fetal development, active VICs are denser, proliferate more and undergo apoptosis more than in adult valves. After birth, these valve cells become more quiescent and the collagen content of the valves matures [18]. However, VICs are activated in valves experiencing abnormal hemodynamic conditions [19] known as fetal development and/or active remodeling (e.g.,
during the Ross procedure) and express more SMC-like (i.e., α-SMA+) phenotype [20, 21]. These activated VICs (myofibroblasts) begin proliferating [22] and contribute to excessive ECM remodeling, ultimately leading to fibrosis and disease [23, 24]. Contractile properties, leaflet stiffness, and the mechanical environment of the valve have been correlated with expression of the SMC-like phenotype in some studies [25, 26]. VEC, while similar in many respects to other vascular endothelial cells, express certain unique phenotypic and morphologic attributes [27, 28].

Typically the complex structure of the valve leaflets (Fig. 6 A) withstand $3 \times 10^9$ cycles (and 80 mmHg pressure in the aortic side and (40 mmHg in the pulmonary side) during an average human lifespan. However, disruption of the valve tissue architecture and organ-level structures, either by congenital malformation or acquired by disease, can lead to clinical symptoms mandating valve replacement.

### 1.2 MECHANICS, STRUCTURE AND FUNCTION OF NATIVE HEART VALVES

Semilunar valve leaflets which passively undergo large deformations in response to the pulsatile blood pressure and flow (fluid shear stress) are unique in their structure, ECM microstructure and mechanical characteristics (Fig. 6 B-C). Their gross anatomical structure, extracellular ECM architecture, and associated mechanical characteristics appear to fulfill physiological functional requirements (e.g., a dense network of circumferentially oriented collagen fibers supports the diastolic transvalvular pressure). The relationships between collagen network structure and leaflet tissue mechanical behavior have been studied extensively [29]. As shown in Fig. 6 B, ECM fibrous alignment defines the anisotropic mechanical characteristic of heart valve leaflets. Dense collagen bundles and elastin network, especially in the fibrosa layer, orient in the circumferential direction causing the leaflets to be stiffer in the circumferential direction and more deformable in the radial direction according to leaflet rearrangement during diastole. Observations of these structural features and biomechanical portents in native valves suggest a potential role for mechanical factors in the development of TEHV. Due to the crimped structure of collagen fibers in the native leaflets (both pulmonary valve (PV) and aortic valve (AV)), less force was required initially
to stretch the leaflets up to the point where the collagen fibers were straiten (up to approximately 15-20% strain). This part is known as the initial region of the stress-strain curve. Following the transient region, where the fibers were completely straight, higher force was required to further deform and stretch the leaflets, which resulted in a sudden increase in the slope of the stress-strain curve and a steeper trend of the stress-strain curve in the peak region (Fig. 6 C).

Figure 6: Leaflet unique anatomical structure (A) [30] and aligned collagen and elastin arrangement in the complex structure (B) [31] of the layers dictates the anisotropic mechanical properties of the heart valve leaflet tissue (C) [32].

1.3 VALVULAR DISEASE AND CURRENT SURGICAL INTERVENTION

The current approach toward heart valve disease treatment includes the heart valve leaflet replacement with biologically-derived or mechanical artificial heart valve. Those indeed improved the quality of life of millions of patients worldwide since 1960’s. In cyanotic congenital heart disease, the
lesions allow for a mixture of oxygenated and deoxygenated blood to be pumped into the systemic circulation, thus resulting in a bluish coloration. The overall incidence of severe cyanotic congenital heart disease has been estimated at 1,391/million live births [33]. Based on a recent US Center for Disease Control (CDC) report of 4,089,950 births [34], this translates to approximately 5,689 new cases of severe cyanotic congenital heart disease per year in the US alone. However, while currently available prosthetic valves (Fig. 7) perform sufficiently well in the short term, the side-effects of anticoagulation therapy (mechanical valves) and the structural degeneration of bioprosthetic and homograft valves represent significant drawbacks in the long-term [35-39]. Especially pronounced in pediatric patients suffering from congenital valvular lesions, the current non-viable devices are limited as none of the available prosthetic valves has the capacity to grow in tandem with the somatic growth of the patient. Thus for the pediatric population there is demonstrated clinical motivation for the development of a valved conduit that can grow with the patient, potentially mitigating the need for subsequent reoperations. One potential approach being investigated is the tissue engineered heart valve (TEHV).

Figure 7: Currently available heart prosthetic valve replacements.
1.4 TISSUE ENGINEERED HEART VALVES

Tissue engineered heart valves (TEHV) have the capability of self-repair and growth due to the existence of living cell population and potentially resistance to infection. A typical TEHV approach is to seed natural [40, 41] or synthetic biocompatible scaffolds with cells [42-48], culture them in static in vitro environments or in bioreactors simulating tissue growth with physiological hemodynamics [49-51], and then implant the cell-seeded constructs in vivo. [44, 52, 53] (Fig. 8). The first study to investigate replacement of a single pulmonary valve leaflet with a tissue engineered leaflet was in 1995 by Shinoka et al. [53] in the laboratory of Dr. John E. Mayer, Jr. TEHV are typically constructed by seeding cells onto a porous, three-dimensional scaffold that resembles the anatomy of a native heart valve [37]. While living, autologous tissue engineered heart valves have been investigated as an alternative to homografts, their function in long-term large animal studies has been suboptimal due to limitations associated with the scaffold. Perhaps, with the exception of electrospun poly(ester urethane) urea (PEUU) scaffolds [54].

Previous scaffolds for TEHV have neither exhibited the anisotropy (i.e., directionally dependent mechanical properties) and elasticity (i.e., ability to reversibly undergo large deformations) of native leaflets nor promoted native-like collagen orientations without the application of in-vitro or in-vivo mechanical loading. In particular, limitations of previous scaffolds included permanent inelastic deformation and high stiffness (nonwovens) [50, 55, 56], small pores inhibiting cell seeding (electrospuns) [47, 57], homogenous structural-mechanical properties (fibrin gels) [41, 42, 58], and immunogenicity (decellularized xenograft valves).

There is a large degree of disparity in the types of materials used for the scaffold. Due to the acute mechanical strength criteria for valve replacements, the majority of TEHV developed thus have relied on synthetic polymeric materials; such as polyglycolic acid (PGA) and poly-L-lactic acid (PLLA) [44, 52, 59], or on natural based materials [40, 41], such as small intestinal submucosa (SIS) [60], and decellularized porcine valves [61]. Both TEHV design paradigms have demonstrated significant early achievements, including the successful demonstration of TEHV function in the pulmonary circulation of a
growing lamb for 120 days (SMC + EC; PGA scaffold) by Hoerstrup et al. [52], and recently a 1 year follow-up on the first TEHV implanted in a human patient (EC; decellularized homograft) by Dohmen et al. [62]. Sutherland et al. [59] demonstrated that TEHV fabricated by seeding bone marrow-derived mesenchymal stem cells onto nonwoven 50:50 blend PGA/PLLA scaffolds could successfully function in the ovine pulmonary circulation for up to 240 days. However, the structural-mechanical properties of nonwovens are not well suited to generating biomimetic leaflet materials. Engelmayr and Sacks found that the micromechanics of nonwoven scaffolds are highly complex, yielding large increases in stiffness with the accretion of relatively small quantities of collagen [50, 55]. Because the PLLA component of 50:50 PGA/PLLA nonwoven scaffolds degrades slowly (~2yrs), leaflets fabricated from such scaffolds become too stiff following implantation. This was evidenced in a more recent study of bone marrow-derived mesenchymal stem cell-seeded TEHV based on nonwoven scaffolds by Gottlieb et al. [44] in which in vivo monitoring of TEHV function demonstrated progressively worse regurgitation from 6 to 12 weeks implantation. This finding was attributed to leaflet stiffening and immobility. Thus tissue engineered constructs must exhibit tissue-like functional properties, with the capability of closely matching the structural-mechanical properties of native heart valve leaflets.
1.4.1 Valvular Interstitial Cell Properties and Implications for TEHV Cell Sources

Native heart valve leaflets are capable of functioning for the $3 \times 10^9$ heart beats of an average human life span due to the homeostatic collagen network self-repair effected by their resident VIC [17]. Notably, recent studies have investigated the distinct phenotypes exhibited by VIC isolated from aortic versus pulmonary valve leaflets, demonstrating, for example, intrinsic differences in force generation [64] and VIC stiffness and collagen synthesis [26, 65]. In selecting a cell source to recapitulate such specific functions in a TEHV, it is important to consider how closely a particular cell type mimics the native VIC phenotype. An important milestone toward a rational basis for TEHV cell source selection was the demonstration by Latif et al. [66] that human bone marrow-derived mesenchymal stem cells (MSCs) and VICs share a very similar molecular expression fingerprint. These phenotypic similarities paralleled similarities in extracellular matrix synthesis observed by Ku et al. [67], who showed similar collagen up-regulation in response to (14%) stretch. The importance of considering calcification potential, and closely matching the phenotype of VICs, was further highlighted by the work of Ferdous et al. [68], who recently demonstrated that VICs exhibited a lower intrinsic calcification potential than vascular smooth muscle.
cells. In particular, VICs could be considered an ideal cell type for forming biomimetic heart valve leaflet tissue, not for their clinical applicability, but rather due to the natural role they play in modulating native valvular tissue remodeling and homeostasis. Thus, in designing clinically-applicable tissue engineered heart valves, one must in general rely on the capacity of less invasively accessible stem or progenitor cells to exhibit a pluripotency suitable for recapitulating a functionally equivalent valvular tissue architecture.

1.4.2 Cellular and Tissue Guidance Effect on TEHV Biomechanics

The potential role of cell and collagen network orientation in mechanical properties of TEHV that associate with heart valve leaflet function has been discussed [9, 11, 43, 69]. Thus, cell secretion and organization of collagen is the most important factor to be controlled in heart valve tissue engineering [43]. Previous TEHV, however, failed to develop significant collagen fiber alignment without either in vivo [70] or in vitro [71] mechanical conditioning and it remains unclear how such conditioning regimens will affect a VIC-based TEHV, which would make the most logical and useful benchmark for optimizing clinically relevant cell sources. Indeed, it was demonstrated previously by Engelmayer et. al. [43], on non-degradable microfabricated epoxy resin scaffolds, that the aspect ratio of rectangular pores can be used to guide both cell and collagen fiber orientation. Moreover, Engelmayer et al. [72] aimed to guide the orientation of heart cells on microfabricated accordion-like honeycomb scaffolds recently using scaffolds structural design. In our most recent study, we observed preferential fibroblast orientation along the long-axis of the diamond-shaped pores of computationally designed microfabricated PGS scaffolds.

1.5 MATERIAL, MICROSTRUCTURE, FABRICATION & MECHANOTRANSDUCTION IN TEHV

Cell attachment to scaffolds for engineered tissue is the basic factor to consider in tissue engineering. This happens via specific interactions between serum proteins adsorbed to the scaffold material or growth factor that scaffolds have been treated with membrane-bound receptors [85]. The porosity of the scaffold also plays an important role in successful cell attachment procedure. Scaffolds with very small pores (nano-fibrous scaffolds) inhibit the cell penetration through the construct and which
mainly results in surface cell attachments. On the other hand, scaffolds with high porosity (greater than 90%), cause a large proportion of the seeded cells to initially adhere to themselves rather than the scaffold. Recently, studies have demonstrated that N-cadherin can facilitate intercellular fibroblasts signaling by calcium-permeable channels which are stretch sensitive [73, 74]. Therefore, considering that and the effect of mechanical stimulation on the cellular behavior (described in next section) the scaffold’s stiffness and mechanical properties are important in further tissue formation and guidance on the scaffolds. For instance, the PGA and PLLA fibers which were widely used for TEHV scaffolds are not elastomeric and it is possible that mechanical characteristic of those polymers are transduced to the cells during the cellular network regeneration and deformation.

Some tissues functionality relies on their structure and architecture, thus it is important to obtain cellular alignment and ECM formation similar to native structure even after scaffolds degradation in vivo or in vitro in engineering of those tissues. Myocardial tissue including various collagen architecture through the ventricular wall and heart valve leaflet with fibrous structure on the surfaces interconnected with gelatin-like component in the middle layer are the examples of these tissues [75, 76]. Recapitulating these complex arrangements to create a functional tissue is a big challenge in tissue engineering. Native leaflets are among those tissues that their functionality depends on their complex structure (i.e., cellular alignment and ECM directions) which defines the architectural arrangement and anisotropic mechanical characteristic of the leaflets. Thus, significant studies attempted to recapitulate leaflet structural complexities in an in vitro setting while providing an appropriate environment for cellular growth and ECM generation [44, 52] during fabrication of scaffolds for TEHV based on synthetic or naturally based scaffolds for TEHV [40, 42, 45, 46].

Following scaffold seeding, ECM deposition occurs during the TEHV cultivation and preferably organized protein structure should fill the void spaces in the porous scaffolds [16, 77].

Different techniques including histology, scanning (SEM) and transmission (TEM) electron microscopic techniques are traditionally employed to observe the presence and growth of tissue formation
and specifically staining protein (collagen, sulfated glycosaminoglycans (S-GAG), and elastin) in TEHV [52, 78, 79]. A confluent surface with cell orientation in the direction of flow has been demonstrated using SEM on the scaffold cultured in a pulse duplicator flow loop [52]. Also collagen and elastin fiber aggregation has been indicated with TEM of the scaffolds [80]. It is possible through immunostaining to depict the α-actin filament in the scaffolds structure. However, it is critical to consider that the structure of the scaffolds (e.g., fibrous like structure, homogenous constructs or microfabricated scaffolds) affect the results of presentation considerably. Studies have shown that scaffold micro-structure and architecture affect the cellular arrangement directly toward tissue guidance in a preferred direction [75]. The lack of detailed information about TEHV microstructure accounts for a significant gap in understanding.

1.5.1 Scaffold Fabrication Techniques

Scaffolds have been designed and fabricated using different technique to obtain various structure (e.g., fabricated and fibrous scaffolds). Various approaches exist to microfabricate scaffolds to create micro-pores or micro patterns on the engineered tissue to resemble native tissue structure, usually leading to tissue formation alignment. High resolution stereolithography [81], replica molding and laser microablation, were previously used for fabricating appropriate pore shapes and non-woven scaffolds with PGA/PLLA[82]and PGS scaffolds [45, 72] respectively. Elastomeric poly(ethylene glycol) (PEG) scaffolds were fabricated using a digital micromirror device (DMD)-based method which lead to the desired pore shapes with high accuracy and similarly designed scaffolds [83]. Moreover, 3D layer assembly methods using simple or complex technologies have been used following single layer scaffolds microfabrication. This technique resulted in successful generation of 3D construct which guide the tissue formation and exhibit appropriate anisotropic characteristic [75, 81, 83, 84]. Different techniques are available to create fibrous scaffolds, including electrospinning, wetspinning, meltspinning and microfluidic-spinning [85]. In the electrospinning method, the fibers are created by the flow of the polymer subjected to an electrical field between the needle tip and the collector plates. Scaffolds with random [57] or aligned fibers [86] can be obtained through this simple and fast procedure. Depending on
the polymer properties, needle gauge size, electrical field magnitude and the distance of the needle tip to the collector, fiber diameters from 50 nm to 100 µm can be obtained.

The first three-dimensional porous scaffolds fabricated from PGS were made by porogen (i.e., salt particle) leaching, yielding highly compliant PGS foams [87, 88]. Salt leaching however, while capable of grossly controlling pore size in PGS foams, provides little control over scaffold microstructural element and pore shape geometries. PGS has been microfabricated by a variety of approaches, including replica molding [89-92], combined solid freeform fabrication-molding [93, 94], and UV laser microablation (e.g., 248 nm krypton fluoride (KrF) excimer laser [72, 84]. Engelmayr et al. [72] demonstrated that excimer laser microfabricated PGS scaffolds comprised of accordion-like honeycomb pores, could be designed to closely match the anisotropic effective stiffnesses of native myocardium while simultaneously promoting alignment of seeded neonatal rat heart cells. Similarly, in a new study, PEG scaffolds were fabricated with DMD method and layer by layer assembly including different pore geometries and resulted in anisotropic constructs [83].

However, while capable of yielding scaffolds mimicking the mechanical properties and anisotropy of soft tissues, electrospinning remains a fundamentally stochastic process due to low molecular weight of PGS. Due to the presence of fibrous ECM networks in native leaflets structure, it is desirable for scaffolds to be comprised of a fibrous structure which closely match native tissue structure and mechanics. Photo curable, acrylate formulations of PGS [95] have facilitated electrospinning of nanofibrous scaffolds [96, 97] achieved otherwise through core/shell [98, 99] and polymer blending [57, 86] approaches. Toward deterministic design and rendering of scaffold microstructures, recent PGS scaffolds have been made mostly by microfabrication. However, other biocompatible polymers including PGA, PLA, Poly-4-hydroxybutyrate (P4HB) are appropriate polymers in use of spinning techniques and have been excessively used in TEHV. In addition, PCL has a great advantage due to its simple electrospinning fabrication and higher strength in mechanical stiffness while providing a less friendly environment for tissue culturing. Therefore, mixing those polymers resulted in a highly biocompatible
structure that the mechanical strength is enough to withstand the pressure in the site of valve leaflets action in circulation [57]. PGS/PCL electrospun scaffolds (including random and aligned fibers) revealed tunable mechanical properties that matched the native heart valve leaflets stiffness and anisotropy in our study. Thus, the main aim of this study was to fabricate an appropriate engineered tissue with structure that matches the native tissue anisotropic structural and mechanical properties. We adopted appropriate fabrication technique to obtain accurate pore geometries (in microfabricated scaffolds) and aligned fibrous structure similar to leaflets structure.

1.6 FINITE ELEMENT ANALYSIS OF TEHV DEVELOPMENT

Finite element (FE) analyses have been proposed to describe the valve mechanical interaction in relation to the surrounding physiological environment. Specifically, studies aimed to assess the leaflet’s functionality, deformation and stress distribution during leaflet’s opening and closing [100-103]. Some study also used computational modeling to evaluate normal, pathological, and prosthetic valves [103]. Mathematical and theoretical analyses were also developed to understand the mechanics of tissue growth and transformation in native and engineered tissues [50, 55, 104-106]. However, while the model and analysis are generally rigorous and accurate, experimental validation is mostly absent in those studies. However, few studies based on computational or theoretical modeling, were devoted to predict the strength and stiffness of biomaterials used in tissue engineering [51, 107].

In describing the mechanical behavior of a TEHV based on microfabricated PGS scaffolds, the construct could be considered as an elastic material with diamond pores to obtain the effective stiffness of the material following the fabrication or cellular deposition. In mathematical modeling on the other hand, scaffold struts can be approximated as discrete fibers which the arrangement will be defined based on diamond pores angle. In this study we aimed to use the FE analysis and rule of mixture model (corrected for the angle) to predict the PGS microfabricated scaffolds stiffness.
1.7 PHYSIOLOGICAL CONDITIONING OF TEHV

Pulse duplicators are main examples of dynamic bioreactor used for THEV. Most bioreactors designs expect to provide the biochemical and mechanical stimuli in accordance with the physiological conditioning of the native heart valve. The pulse duplicators are mostly used to test commercial valve hydrodynamics (prosthetic valves) [108-110]. Although many types of bioreactors have been used for conditioning of TEHV, most of those generate a pulsatile flow medium through the system in order to provide mechanical conditioning and nutrient supply [111-113]. Thus, bioreactors provide an environment to condition cell-seeded scaffolds in an in vitro environment within which flow rate, pressure, temperature, and oxygen diffusion should all be controlled to provide a physiologic environment [112-114] to derive extracellular matrix formation and cellular alignment. However, providing a system which completely mimics the heart circulation while maintaining the above conditions is extremely difficult [115]. We believe with the use of bioreactors we will be able to provide mechanical stimulation on TEHV using physiological conditioning. In this study we aim to design a novel stretch/flexure bioreactor which will apply physiological stimulation during TEHV cultivation, to study the effect of mechanical stimulation on scaffolds physical characteristic and tissue formation.

The effect of TEHV pre-implantation in bioreactors were compared to static incubation and the more robust tissue formation were evident in the TEHV cultured in bioreactors [78, 111, 116, 117] (Fig.9). In addition, several limitations are associated with pulse duplicators and flow loop bioreactors with their useful role in TEHV development for implantation. Because these bioreactors aim to mimic the complex physiological in vivo environment [49, 118], it is difficult to study the effect of individual mechanical stimuli such as shear stress, pressure, tension, and flexure as they are inextricably coupled in previous system. Therefore, it would be difficult to understand the individual contribution of any particular mechanical factor in an intact TEHV.

Toward understanding the specific effect of each mechanical stimulation mode in TEHV development, bioreactors have recently been developed to provide fluid shear stress [115, 119-121],
cyclic flexure [78, 111], and tension [112, 122] to TEHV biomaterials. While comparative results between static and bioreactor conditioning in this study have provided extensive information for the individual role of simple modes of mechanical stimulation in TEHV development, optimization of those aspects requires an understanding of the relevant cellular mechanisms.

Figure 9: Bioreactors developed to study (A-B) the effect of shear flow [112], (C) cyclic stretch [78] and (D) cyclic stretch, flexure and flow individually [111].

1.7.1 Effect of Mechanical Stimulation on Cells and Tissue Regeneration

The critical role of mechanical forces on the homeostasis and regeneration of many organs that are subjected to physiological loading \textit{in vivo} has been studied previously [123-125]. Similar studies were conducted to evaluate tissue development in native and engineered tissues under mechanical stimulation using \textit{in vitro} settings [113, 126-129]. Thus, systems were developed to study
the effect of mechanical stimulation based on their relevant application and physiological conditions that the load bearing tissue are exposed [122, 130]. Leung et al. [131] was the first group that demonstrated the effect of cyclic mechanical stretch on ECM synthesis in vitro. They established a bioreactor in which rabbit aortic smooth muscle cells (SMCs) were stretched on an elastin membrane derived from the media layer of bovine aorta and lead to the 3-5 fold increased in type I and type III collagen biosynthesis compared with static or agitated controls with the application of cyclic stretch at an amplitude of 10% and frequency of 1 Hz (all in the absence of any change in SMC proliferation). Engelmayr et al. [78] previously demonstrated a positive linear relationship between engineered tissue modulus and bulk collagen concentration in bioreactor studies comparing cyclic flexure to static culture conditions. The continuous laminar flow in their bioreactor (applying the fluid shear stress) resulted in promoting hydroxyproline deposition (a marker for collagen) in TEHV reconstruction following 14 days. They also demonstrated that cyclic flexure and laminar flow could synergistically accelerate collagenous tissue formation seeded by bone marrow derived MSCs [111]. In addition with those effects, cyclic flexure replicated many of the aspects of TEHV development in pulse duplicator bioreactors, including a nearly two-fold increase in collagen growth compared to static controls, and strong expression of the intermediate filament vimentin (marker associated with native valve cell phenotype). Further, Engelmayr et al. [72] previously demonstrated that microfabricated PGS scaffolds can withstand cyclic stretch in vitro. Using commercially available devices like Flexercell® allowed them to investigate the effect of the mechanical stimuli including; cyclic strain amplitude [131-133], and strain rate [134, 135], on cell phenotype, proliferation [76-78], apoptosis [136, 137], and ECM generation.

1.8 MOTIVATION AND AIMS OF THE PRESENT STUDY

TEHVs, by virtue of their potential for growth, remodeling and resistance to infection, may be useful in the repair of congenital or acquired valvular lesions. Given the relative success of cryopreserved homografts and the established dependence of leaflet function on collagen network organization, it is
important that scaffolds for the functional tissue engineering of heart valve leaflets be capable of mimicking native leaflet mechanical properties at the time of implantation while guiding native-like cell and collagen fiber orientations as the engineered tissue remolds toward a scaffold-free, self-reliant state. Moreover, the ability to reversibly undergo large strains can help to promote and guide tissue growth. However, there are drawbacks including unnatural large stiffness, lack of anisotropic characteristics, large pore sizes, unnatural plastic deformation and inability to be sutured that prevented these scaffolds from being successful translated to in vivo models.

To improve on existing design and address current limitations, the main objective of the studies described herein was to develop scaffolds capable of mimicking the structure and anisotropic mechanical characteristics of native leaflets while promoting native-like cell and collagen content and ECM (e.g., collagen) content and organization. Herein we designed scaffolds based on biodegradable elastomer (PGS) and PCL polymers, individually or in combination and employed fabrication techniques to obtain appropriate structure and mechanics for TEHVs. Various fabrication techniques, including laser microablation, micromolding, and electrospinning were adopted based on the most feasible fabrication method for scaffolds that mimics a native tissue leaflet’s structure and mechanics. Another objective is to determine the capacity of various cell types, including porcine pulmonary and aortic VICs, human VICs, and sheep MSCs and VICs to form a biomimetic engineered heart valve tissue on designed scaffolds. We were able to study the potential of the above cell sources in mediating native-like tissue formation on the constructed scaffolds and compared the cellular behavior with respect to the scaffold’s structure in a more detailed study. Based on the preliminary study on microfabricated PGS scaffolds and microfibrous PGS/PCL scaffolds, and evaluating tissue regeneration with different cell sources, we further hypothesized that combining PGS/PCL microfibers and microfabricated PGS in a tri-layered construct provided elastic and anisotropic characteristics for the 3D architecture which matched structural and mechanical properties of native leaflets while simultaneously supporting controlled cellular growth and tissue formation within a guided architecture. This approach could have the potential for successful translation towards a tissue engineered heart valve replacement.
In addition to developing clinically-applicable engineered heart valve tissues, we aimed to systematically quantify and compare the effects of static versus cyclic stress (and/or alternatively flexure) on the development of the ECM generation and cellular orientation in the TEHV by culturing the fabricated scaffolds in a novel designed stretch/flexure bioreactor. This information is critical for developing *in vitro* conditioning regimens to optimize the material properties of the TEHV prior to implantation. Our overall objective is that such “ideal” engineered heart valve tissues (i.e., based on MSCs) will complement native heart valve leaflet tissue in providing benchmarks for use in the design of clinically-applicable TEHV.

We combined VICs and MSCs with microfabricated PGS scaffolds and microfibrous PGS/PCL scaffolds to create a more biomimetic tissues for TEHV. We aimed to design a bioreactor with relevant mechanical conditioning to study the effect of mechanical stimuli on tissue generation. The achievements of the study have been summarized in the following.

1: **Design VIC and MSC-seeded scaffolds to mimic heart valve leaflet structure and mechanical properties.**

In this study, various fabrication techniques including laser microablation, electrospinning and micromolding were applied individually or in combination to fabricate TEHV scaffolds exhibiting the appropriate structure and associated anisotropy and mechanical characteristics. The polymeric scaffold material-of-construction was chosen among PGS and a combination of PGS and PCL. In the preliminary study, PGS scaffolds were computationally designed and laser microfabricated to match the anisotropy and peak tangent moduli of bovine native heart valve leaflets [45]. We demonstrated that the PGS scaffold’s potential to support tissue formation via fibroblast-seeded scaffolds were successfully cultivated *in vitro* for 3 weeks and assessed for collagen and DNA contents. In addition, FE analysis was used to predict the scaffolds stiffness as a function of pore geometry to optimize the PGS microfabricated design for creating scaffolds with stiffness and anisotropy similar to native characteristic. Two layered microfabricated PGS scaffolds were then assembled to improve the construct’s mechanical properties while providing more 3D-like engineered tissue. This study was conducted with aortic and pulmonary
cells (seeded on scaffolds with low and high density) to evaluate and compare the potential of VICs for tissue formation on fabricated scaffolds. To improve the existing design, we created a tunable and appropriate fibrous structure based on PGS/PCL for TEHV. The effect of fibrous structure and mechanical characteristics of scaffolds were studied on human cellular behavior was evaluated. Furthermore, both fabrication methods were combined to generate scaffolds that mimicked heart valve leaflet’s structure and mechanics. Finally, a novel stretch-flexure bioreactor was designed and fabricated in the lab machine shop to culture the scaffolds under mechanical stimuli.

a. Develop a finite element (FE) model to predict scaffold and cell-seeded construct mechanical properties. To better understand the effect of geometrical variables and fabrication design on the scaffold’s stiffness, we developed a precise and detailed FE model that was compared with theoretical formulation to predict the PGS microfabricated scaffolds stiffness based on geometrical parameters. This helped to optimize the fabrication design for obtaining scaffolds with stiffness and anisotropy resembling native tissue leaflets. We developed 3-D FE models of microfabricated scaffolds comprised of diamond-shaped pores in order to predict their mechanical properties (i.e., effective stiffness and anisotropy). The effective stiffnesses of the scaffold, predicted by the FE model, were in agreement with the experimental results as shown in result section. The FE analysis was applied to computationally design microfabricated PGS scaffolds to match the moduli and anisotropy of native heart valve leaflets. These models will be adapted to predict mechanical properties of microfabricated scaffolds and tissue-scaffold composites from constituent orientations, volume fractions, and stiffness.

b. Develop single-layered and double layered microfabricated TEHV scaffolds and evaluate cell seeding (e.g., with fibroblast and VICs). Single layer PGS microfabricated scaffolds were designed and fabricated using laser microablation and micromolding techniques. Double layered PGS microfabricated scaffolds were then fabricated with a simple assembly method, and cell seeded constructs were compared in terms of ECM deposition and mechanical characteristics. Studies collectively suggest that clinically relevant MSCs are capable of recapitulating the phenotype of native VICs, indicating that a VIC-based TEHV would make a useful benchmark for optimizing the mechanical conditioning of TEHV. Thus, in
these studies, scaffolds were seeded with VICs, and scaffold regeneration and degradation were assessed over a period of pre-determined cultivation times.

c. Fibrous scaffolds and cellular characteristic

One of the most important structural characteristics of mature heart valve leaflets is their intrinsic anisotropy, which is derived from the microstructure of aligned collagen fibers in the extracellular matrix. In the present study, we used a directional electrospinning technique to fabricate fibrous PGS:PCL scaffolds containing aligned fibers, which resemble native heart valve leaflet ECM networks. In addition, the anisotropic mechanical characteristics of fabricated scaffolds were tuned by changing the ratio of PGS:PCL to mimic the native heart valve’s mechanical properties.

d. Develop a tri-layered engineered tissue for designing a tissue engineered leaflet that match the structural-mechanical properties of native heart valve leaflets.

Fabrication of tri-layered scaffolds, by assembling the microfabricated PGS scaffolds and microfibrous layers of PGS/PCL was pursued to move toward improving the structure and mechanics of designed TEHV. We electrospun the fibers directly on the middle layer (microfabroicated PGS layer) and applied plasma treatment to assemble the tri-layered constructs. Due to the improved pore interconnectivity and 3-D structure, we anticipated that tri-layered scaffolds would yield enhanced tissue formation capable of more closely resembling the structural, mechanical and functional properties of native heart valve leaflets. More importantly, we found that those scaffolds guided the tissue formation in the preferred circumferential direction.

2. Design simple stretch and flexure bioreactor to study the effects of static and cyclic stretch on collagen synthesis VIC-seeded PGS scaffolds. In order to complete studies associated with this aim, we designed a novel bioreactor capable of applying cyclic stretch and/or flexure to biomaterial samples and cell-seeded scaffolds. The strain rate would be correlated to match the physiological conditions. VIC-seeded microfabricated PGS scaffolds with diamond shaped pores were subjected to cyclical stretch and flexure in the bioreactor. Collagen contents and cellular orientation were quantified.
Microfabricated PGS scaffolds may be applicable to tissue engineering blood vessels, heart valve leaflets, and myocardium. In the current study, PGS scaffolds were computationally designed and microfabricated by laser ablation to match the anisotropy and peak tangent moduli of native heart valve leaflets. Finite element simulations predicted PGS curing conditions, scaffold pore shape, and strut width for matching the scaffold effective stiffnesses to the leaflet peak tangent moduli. Based on simulation predicted effective stiffnesses of 1.10 MPa and 0.18 MPa for the scaffold preferred (PD) and cross-preferred (XD) directions, scaffolds with diamond-shaped pores were microfabricated by laser ablation of PGS membrane, cured 12 hours at 160°C. Effective stiffnesses measured for the scaffold PD (0.83 ± 0.13 MPa) and XD (0.21 ± 0.03 MPa) were similar to predicted values and peak tangent moduli measured for bovine aortic valve leaflets in the circumferential (1.00 ± 0.16 MPa) and radial (0.26 ± 0.03 MPa) directions. Scaffolds cultivated with fibroblasts for 3 weeks accumulated collagen (736 ± 193 µg/g wet weight) and DNA (17 ± 4 µg/g wet weight). This study provides a basis for the computational design of biomimetic microfabricated PGS scaffolds for tissue engineered heart valves and cardiovascular tissue engineering applications.

2.1 INTRODUCTION

Advanced bioprosthetic and mechanical replacement heart valves, while functionally inferior to their healthy native counterparts, are both lifesaving (10-20 years) in adults suffering from valvular disease. Such advances in valve replacement, however, have yet to be effectively translated to neonates and young children suffering from congenital valvular lesions. While cryopreserved valve homografts, the current gold standard for pediatric valve replacement, are lifesaving, children may require as many as five reoperations prior to adulthood to accommodate increases in ventricular outflow tract diameter with somatic growth [138]. Toward improving the quality of life of these young patients, investigators are pursuing the development of living, tissue engineered replacement TEHV [37].
Early TEHV were fabricated in vitro by cultivating autologous, vascular-derived cells on nonwoven PGA scaffolds. First developed in the laboratory of Dr. John E. Mayer, Jr. at Children's Hospital Boston [53], the potential of this prototypical TEHV paradigm has since been demonstrated in several large animal studies. Notably, Hoerstrup et al.[52] demonstrated 5-month function as pulmonary valve replacements in growing lambs, correlated with functional maturation of the TEHV cellular phenotypes and extracellular matrix composition and organization demonstrated by Rabkin et al.[16]. Toward clinical translation, Sutherland et al. [139] demonstrated long-term function of TEHV constructed by seeding similar nonwoven textile scaffolds with relatively noninvasively accessible bone marrow-derived mesenchymal stem cells. Recent advances by Gottlieb et al.[44] have further refined the original paradigm while simultaneously highlighting one of its principal limitations: the high stiffness, inelasticity, and complex micromechanics [55, 82] of the needle-punched nonwoven scaffold. Toward designing scaffolds with improved elasticity and structural-mechanical anisotropy similar to native semilunar valve leaflets [140], we introduced an electrospun poly(ester urethane urea) scaffold exhibiting a biaxial mechanical response similar to native valve leaflet tissue. The processes of fabricating and characterizing these electrospun elastomeric scaffolds were further refined by [141], including accounting for the influence of “micro-integrated” particles (e.g., cells), as introduced by [142]. Electrospinning, however, is an inherently stochastic process in which control of microstructural morphology is possible only in an average sense [143]. First investigated the utility of PGS in the context of TEHV, demonstrating the ability of peripheral blood-derived endothelial progenitor cells to proliferate and synthesize extracellular matrix within PGS foam scaffolds coated with various cell adhesive proteins. A limitation of PGS foam, however, is its low stiffness (4-8 kPa) compared with native valve leaflet tissues and effectively random pore structure.

Engelmayr et al. [72] demonstrated that excimer laser microfabricated PGS scaffolds comprised of accordion-like honeycomb pores could be designed to closely match the anisotropic effective stiffnesses of native myocardium while simultaneously promoting alignment of seeded neonatal rat heart cells. To facilitate scaffold design, Jean and Engelmayr developed periodic finite element models capable
of retrospectively predicting the effective stiffnesses and anisotropy of accordion-like honeycomb PGS scaffolds [107]. In the current study, PGS scaffolds were computationally designed and microfabricated to match aspects of the structural mechanics of native semilunar heart valve leaflet tissues.

2.1.1 Aim and Hypothesis

The overall goals of the current study were (i) to investigate the hypothesis that computationally designed PGS scaffolds microfabricated by laser ablation can be generalized to cardiovascular tissues other than myocardium and (ii) to demonstrate, for a different pore design and laser, that our finite element model could accurately predict measured scaffold mechanical properties. As a clinically important example application, PGS scaffolds herein were computationally designed and microfabricated to match aspects of the structural mechanics of native semilunar heart valve leaflet tissues. Mechanical properties of native bovine aortic valve leaflet tissue were measured by uniaxial tensile mechanical testing of circumferentially and radially oriented tissue strips. Using a diamond-shaped pore structure previously shown to be capable of guiding engineered tissue collagen orientation [82], periodic finite element simulations [107] were used in conjunction with a standard curve of PGS stiffness versus curing time at constant temperature [72] to guide the choice of PGS stiffness, diamond-shaped pore geometry, and PGS strut dimensions. Computationally designed scaffolds were microfabricated by 213 nm laser ablation of PGS [84, 144] and characterized by scanning electron microscopy and uniaxial tensile mechanical testing. As an initial demonstration of their potential to promote collagenous tissue formation, PGS scaffolds comprised of diamond-shaped pores were seeded with fetal rat dermal fibroblasts previously used to evaluate scaffold-mediated cell and collagen orientation [82] and cultivated for three weeks in vitro. Cell seeded constructs were assessed by laser scanning confocal microscopy of constructs fluorescently labeled for filamentous (F)-actin and for DNA and collagen content by biochemical assays.
2.2 MATERIALS AND METHODS

2.2.1 Scaffold Geometry and Computational Finite Element Analysis

2-D periodic finite element models were developed using Z-set software (Northwest Numerics and Modeling, Inc., Coventry, RI) as in our previous finite element analyses of accordion-like honeycomb PGS scaffolds[107]. In the simulations, the Young’s modulus of the 160°C / 12h PGS struts (assumed linear elastic based on previous mechanical characterization[72]) was prescribed to be 2.1 MPa (based on uniaxial tensile tests of PGS membranes; Fig.10).

![Figure 10: Effective stiffnesses (E; equivalent to Young’s moduli) measured for solid PGS sheets cured for different times (8, 12, and 16 hours) at 160°C under < 50 mTorr vacuum. For comparison, results obtained previously under equivalent curing conditions by Engelmayr et al.[72](MIT PGS) are presented side-by-side with results obtained in the current study (Penn State PGS). Note the similar trend of increased E with increased curing time at constant temperature.](image)

The scaffold was defined as a tessellation of diamond-shaped pores of the same size and same geometry (e.g., 2:1 aspect ratio) separated by PGS struts of 50 microns width. The diamond-shaped pores were spatially distributed according to vectors of periodicity. The two primary vectors of periodicity,
\( v_1 \) and \( v_{II} \) are depicted in Fig. 11A and were coded in C++ as a function of the inside strut length \( l \), the strut width \( w \), and the acute interior angle \( \alpha \):

\[
(1-1) \quad v_1 = (w+l) \cos \left( \frac{\alpha}{2} \right) x - (w+l) \sin \left( \frac{\alpha}{2} \right) y
\]

\[
(1-2) \quad v_1 = (w+l) \sin \left( \frac{\alpha}{2} \right) x + (w+l) \cos \left( \frac{\alpha}{2} \right) y
\]

The scaffold geometry was constructed (i.e., the coordinates for the center of each diamond-shaped pore were defined) by translating the center coordinates of diamonds belonging to the tessellation with respect to a central diamond (i.e., the diamond labeled 1 in Fig. 11A) along translation vectors \( t \) calculated from combinations of the two primary vectors of periodicity:

\[
(1) \quad t = Av_1 + Bv_2
\]

As depicted in Fig. 11A, the central diamond had 4 first neighbors (i.e., having a common edge) and 4 second neighbors (i.e., having a common vertex). The coordinates of the 4 first neighbors were obtained from Equation 3 by setting the values of parameters \( (A,B) \) to \((0,1), (1,0), (0,-1) \) and \((-1,0)\); for the 4 second neighbors, the values of parameters \( (A,B) \) were set to \((1,1) \) (yielding the diamond labeled 2 in Fig. 11A), \((-1,1) \), \((-1,-1) \) and \((-1,-1) \). Additional diamonds beyond the first and second neighbors were likewise added to the tessellation by permutating parameters \( A \) and \( B \) among appropriate integer value combinations. In \( \mathbb{Z} \)-set, the diamond unit cell (periodic computations) or tessellation (kinematic uniform boundary conditions) was meshed using a Cartesian discretization of square elements with a quadratic interpolation of the displacement (2028 elements and 6189 nodes per diamond unit cell). Computations were conducted on an Apple MacPro desktop workstation with two quad-core 2.93 GHz Intel Xeon processors, 32GB of 1066 MHz RAM, 1 TB of hard drive storage, and 4 NVIDIA GeForce GT 120 512 MB graphics cards. A detailed description of the periodic finite element analysis methods can be found in Jean and Engelmayer [107] in detail.
Figure 11: **Computational modeling of PGS scaffolds;** (A) Schematic illustrating a representative tessellation of 13 diamond-shaped pores (i.e., unit cells) used in finite element simulations to predict the effective stiffness of microfabricated PGS scaffolds using Z-set software. The central diamond (labeled 1) has four first neighbors (i.e., sharing a common edge; demarcated by a blue line). Primary vectors of periodicity $\mathbf{v}_I$ and $\mathbf{v}_II$ used in generating the set of unit cell coordinates for building the tessellation are indicated. Representative 2-D periodic finite element stimulations of the diamond-shaped unit cell deforming in response to an applied strain of 50% in the scaffold (B) preferred (PD) and (C) orthogonal, cross-preferred (XD) material directions. Note that the red wire-frame indicates the undeformed unit cell shape. Color scale bar indicates the magnitude of von Mises stress (red = higher; blue = lower).

### 2.2.2 PGS Pre-polymer Synthesis and Curing

PGS pre-polymer was synthesized by adapting previously described methods [145]. Specifically, 113.8 g of anhydrous glycerol (Product # 49770; Sigma-Aldrich, Corp., St. Louis, MO) and 202.25 g of sebacic acid (Product # 28,325-8; Sigma) (i.e., 1:1 molar ratio) were charged into a 1000 ml 3-neck round bottom flask (CG-1522-07; Chemglass Life Sciences, Vineland, NJ). The reactants were magnetically stirred at 200-400 RPM (Standard 10x10”; VWR, West Chester, PA) and heated under a dry nitrogen blanket to 120°C using a heating mantle (Model 100A O408; Glas-Col, LLC, Terre Haute, IN) and digital temperature controller (Model 210; J-KEM Scientific, Inc., St. Louis, MO). Following 24 h, the nitrogen line was removed and the flask was connected via a distillation adaptor (CG-1022-01; Chemglass) to the inlet of a Liebig condenser (CG-1218-01; Chemglass). The outlet of the Liebig condenser was connected
via a vacuum take-off adaptor (CG-1050-01; Chemglass) to a 250 ml single-neck round bottom flask for collecting condensate (CG-1506-17; Chemglass). Vacuum (< 50 mTorr; Model 1405; Welch Vacuum Technology, Niles, IL) was applied via the vacuum take-off adaptor for an additional 24 h to yield a viscous PGS pre-polymer which was decanted into a clear glass storage jar and solidified into a soft waxy consistency upon cooling to room temperature. PGS pre-polymer was stored in a dessicator at room temperature prior to use.

Methods for making thin (~250 µm) sheets of cured PGS were adapted from previous studies[72, 146]. In brief, standard glass microscope slides (75 x 25 x 1mm; VWR) were first soaked overnight in 0.5M NaOH (Sigma) to improve wettability, rinsed exhaustively in distilled water, and then coated with a thin layer of 90% (w/v) sucrose in distilled water using a custom-built spin-coater (3000 RPM; 30 s). Sucrose-coated slides were then baked in an oven at 120°C for 18 h prior to use. To target a PGS thickness of 250 µm, 0.5 g of PGS pre-polymer was melted and uniformly spread on a sucrose-coated slide. PGS pre-polymer coated slides were then cured in a vacuum oven (Model 1470; VWR) under high vacuum (< 50 m Torr; Model 1405; Welch Vacuum Technology) at 160°C for times ranging from 8-16 h to yield PGS with different mechanical properties [29]. Following curing, PGS sheets were left adhered to sucrose-coated glass slides and stored in a dessicator at room temperature. Prior to use, PGS sheets were delaminated from sucrose-coated glass slides by soaking for 24 h in distilled water, soaked an additional 24 h in 70% ethanol to help remove unreacted monomers [56], and then rehydrated in distilled water prior to laser microablation or mechanical testing. **Fig.12** demonstrates the experimental set up PGS synthesis in Penn. State lab.
2.2.3 PGS Scaffold Fabrication by Laser Microablation

PGS scaffolds were microfabricated by adapting methods previously developed by Engelmayr et al. [72] on a 248 nm KrF excimer laser microablation system (Rapid X 1000; Resonetics, Nashua, NH) to a 213 nm solid-state, frequency quintupled Nd:YAG laser microablation system (LSX-213; CETAC, Inc., Omaha, NE) [84, 144]. We were able to create different pore geometries and structure using custom made masks (Fig.13). The laser machine is running with correlated software which helps to define the initial point of ablation and number of pores and shots to create the arrays of structure. In order to pattern the desired pore tessellation, a custom program was developed using Microsoft Excel Visual Basic for Applications (VBA), as developed previously for generating accordion-like honeycomb pore patterns on the LSX-213 [82]. The custom program allowed the user to input the size of the scaffold (in terms of rows and columns of pores), the strut width, and lasing parameters. Upon execution, the VBA program output a text file of coordinates and lasing parameters formatted appropriately for uploading into the Digilaz II software (v3.1.0; CETAC, Inc.) controlling the LSX-213 laser microablation system. One of the
disadvantages of this method apart from its cost is the time consuming procedure. Fabrication of a small piece of 1×1 cm scaffolds (containing diamond pores with the length of 250 µm) would take about 3 hours to be completed.

Figure 13: Preliminary results on scaffold microfabrication and cell seeding. (a-c) Environmental scanning electron micrographs (ESEM) of PGS scaffolds exhibiting a variety of diamond-shaped pore geometries were (d) microfabricated in Penn. State using an LSX-213 laser microablation system; CETAC, Inc., Omaha, NE). (e) DIC micrograph of a fibroblast-seeded scaffold showing fibroblast attachment to the PGS struts. (f) ESEM showing fibroblast-mediated tissue formation following 3 weeks culture.

In order to generate diamond shaped pores similar to those previously demonstrated suitable for guiding fibroblast and de novo synthesized collagen orientation on non-degradable microfabricated scaffolds[82], a custom alumina ceramic mask was designed and fabricated (CETAC, Inc.), including diamond shaped apertures of the following long:short axis dimensions (in mm): 3:3, 3:2, and 3:1.5. Following demagnification through the optical components of the LSX-213 system, the resultant long x short axis dimensions of the 2:1 aspect ratio diamond shaped pores ablated through the PGS (obtained via the 3:1.5 mm aperture) were ~240 x 120 microns (e.g., Fig.14). Of note, in describing the directionally-
dependent behaviors of the scaffold, herein the material direction parallel to the pore long axis was termed the preferred direction (PD) and the orthogonal material direction was termed the cross-preferred direction (XD).

Figure 14: Representative ESEM at 100x (A) and 300x (B) of microfabricated PGS scaffolds comprised of 2:1 aspect ratio diamond-shaped pores. Scaffold PD and XD material directions are indicated.

PGS membranes (~75 x 25 mm; ~250 µm thick) were removed from distilled water and flattened out on a flat piece of black rubber (i.e., a standard black rubber stopper) that was pre-wetted using ~2mL of 90% (w/v) sucrose in distilled water solution. The PGS membrane was set aside to partially dry on the rubber for ~15 min prior to situating the assembly onto the x-y-z stage of the LSX-213 system. Laser parameter values used herein were: 50% energy level (i.e., yielding ~2.25 mJ per pulse), 20 Hz pulse frequency, and 300 pulses per pore. Improving upon the fidelity of pore shapes generated previously by focusing the laser on the top surface of the PGS membrane, herein it was determined through pilot studies that defocusing the beam 1000 microns (i.e., positioning the top surface of the PGS membrane 1000 microns above the beam’s focal point) resulted in both a qualitatively higher fidelity rendition of the desired diamond shape, as well as larger pore dimensions. Thus a defocus value of 1000 microns was used throughout the current study. For routine SEM and cell seeding studies, overall scaffold dimensions of ~5 x 5 x 0.25 mm were used; for mechanical tests, dimensions of ~25 x 5 x 0.25 were used.
2.2.4 PGS Sheet and PGS Scaffold Mechanical Testing

Solid PGS sheets cured for different times (8, 12, and 16 hours) at 160°C and laser ablated PGS scaffolds were mechanically tested in uniaxial tension as described above for bovine aortic valve leaflet tissues with the exception of the gripping method. In contrast to the native tissue specimens, which were directly clamped within the tester’s grips, PGS sheet and PGS scaffold specimens were first aligned and affixed to a heavy paper tab (cut out from a standard manila folder) using a hot glue gun (DT-200 Dual Temperature; FPC Corp., Wauconda, IL) and all-purpose mini glue sticks (FPC Corp.) as described previously[82]. The tabs facilitated aligning the specimens within the grips with a uniform gage length.

2.2.5 Native Bovine Heart Valve Leaflet Mechanical Testing

To provide a readily available and physiologically relevant native tissue basis for the design of PGS scaffold mechanical properties, circumferentially (CIRC) and radially (RAD) oriented specimens of native bovine aortic heart valve leaflet tissue were subjected to uniaxial tensile mechanical testing. Bovine hearts from 20-30 month old bulls (Black Angus breed) were kindly donated by Brenneman’s Meat Market (Huntingdon, PA). Hearts were rinsed, inspected, placed in plastic storage bags and maintained on ice following slaughter. Hearts were retrieved within ~2 hours of slaughter and transported ~45 min immersed in an ice cold solution of 2% (v/v) antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA) in Hank’s Balanced Salt Solution (HBSS; Invitrogen) in a cooler packed with ice. Aortic valve leaflets were dissected from each of two hearts, providing 3 leaflets each for cutting CIRC and RAD test specimens (note that no distinction was made between coronary and non-coronary leaflets). Test specimens were cut using a scalpel from the central portion of each leaflet as, on average, 16 mm long x 5 mm wide CIRC or 15 mm long x 5 mm wide RAD rectangular strips (Fig. 15 A). Thicknesses of specimens were measured at 3 equally spaced locations along their length using a dial gauge (accuracy 0.01mm; The L.S. Starrett Co., Athol, MA). Uniaxial tensile stress-strain tests were performed using a screw-driven uniaxial tensile tester (model 5866 mechanical tester; Instron Corporation, Norwood, MA) controlled by Bluehill® 2 software (version 2.9). A 10 N load cell was used for all tests. Native leaflet
tissue specimens were removed from HBBS, positioned and clamped within the tester grips, and tested immediately (i.e., at room temperature and moist but not submerged). After setting the gage length to ~5 mm and initial load to zero, each strip was strained to failure at a displacement rate of 10 mm/min. The peak tangent modulus of each tissue specimen was determined by linear regression on the stress-strain data in the peak (i.e., maximum slope) stress-strain region (specimen and testing direction dependent; e.g., 50-70% strain for a radially oriented specimen of bovine aortic valve leaflet tissue). Ultimate tensile strength (UTS) and strain-to-failure (εf) were taken as the maximum stress and strain measured at the onset of failure, respectively.

Figure 15: (A) Schematic illustrating the preparation of circumferential (CIRC) and radial (RAD) uniaxial tensile mechanical test specimens from bovine aortic valve leaflets. Note that the half-moon (i.e., semilunar) shapes each represent an excised aortic valve leaflet (solid black lines) and that the rectangular shapes each represent a CIRC- or RAD- oriented tissue specimen cut out from the leaflet (dashed black lines). Note that the CIRC and RAD directions of uniaxial stretch are indicated (black arrows). (B) The leaflets has been mounted on sand paper and positioned between two grips to avoid slipping of the samples. (C) Representative uniaxial tensile stress-strain curves for CIRC and RAD stretched specimens of bovine aortic valve leaflet tissue. Associated representative peak tangent moduli (E\text{tangent}), corresponding with the steepest portions of the nonlinear stress-strain curves, are denoted. In the current study, the peak tangent moduli of the native aortic valve leaflet tissues were chosen as the scaffold mechanical design criteria (rather than initial moduli) because it was recognized that the initially linear elastic scaffolds would need to be capable of withstanding peak loads during valve closure.
2.2.6 Cell Culture, Scaffold Seeding and Construct Cultivation

Fetal Sprague-Dawley rat skin fibroblasts (27FR; ATCC # CRL-1213; ATCC, Manassas, VA) were used as a model, collagen-synthesizing cell type as done previously by Engelmayr et al. [82]. Cells were expanded in tissue culture-treated polystyrene T25 flasks (BD Falcon™; BD Biosciences, Franklin Lakes, NJ) in complete medium (i.e., Dulbecco’s modified Eagle’s medium (DMEM) (4.5 g/l glucose; 4 mM L-glutamine; no sodium pyruvate) supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) antibiotic-antimycotic (Invitrogen)). Cells from three confluent T25 flasks (passage 2-6) were trypsinized (0.25% (w/v) trypsin, 1mM EDTA; Invitrogen), pooled, and resuspended in 36 ml of culture medium to yield to a cell suspension of ~2.1x10^5 cells/ml, as determined by cell counts with a hemocytometer. Prior to seeding, microfabricated PGS scaffolds (n=3) were autoclave-sterilized (121°C, 15 PSI, 30 min dwell) and soaked in complete medium for 6 days to help improve cell attachment, as described previously by Chen et al. [147]. The general seeding techniques have been described previously [51, 72, 82]. Each scaffold was placed into an individual, sterile-vented 50 ml bioreactor tube (Product # 87050; TPP Techno Plastic Products AG, Trasadingen, Switzerland), thus allowing for gas exchange during seeding. Cell suspension (12 ml) was pipetted into each of the three bioreactor tubes yielding a seeding density of ~10×10^6 cells/cm². The bioreactor tubes were rotated at 8 RPM for 24 h on a rotisserie (Labquake® hybridization rotator; Thermo Fisher Scientific, Watham, MA) inside a humidified incubator operating at 37°C and 5% CO₂ (NU-8700; Nuaire, Inc., Plymouth, MN). Following seeding, scaffolds in bioreactor tubes were observed under an inverted, phase contrast light microscope to verify cell attachment. Cell-seeded scaffolds were subsequently transferred into individual wells of a 6 well plate (Costar Ultra Low Attachment; Corning, Corning, NY) and cultivated statically for 3 weeks in 4 ml per scaffold of complete medium further supplemented with 82 µg/ml of L-ascorbic acid-2-phosphate sesquimagnesium salt hydrate (Sigma) to promote collagen synthesis [148].
2.2.7 Collagen and DNA Assays

The DNA content of engineered tissue extracts was quantified using the PicoGreensDNA quantitation kit (Invitrogen) per the manufacturer’s instructions as described previously [51, 72] using a Spectramax Gemini XS plate reader (Molecular Devices, Inc., Sunnyvale, CA). For DNA extraction, samples (~2×2 mm) were cut from the cell-seeded scaffolds and weighed (average wet weight 0.02 g) prior to extraction. Samples were transferred into microcentrifuge tubes and enzymatically digested in 1ml of buffered 0.125 mg/mL papain solution for 10 h in a 60°C water bath to yield engineered tissue extracts suitable for the PicoGreen assay. The collagen content of engineered tissue extracts was quantified using the Sircol™ collagen assay kit (Biocolor Ltd., United Kingdom) per the manufacturer’s instructions using a Genesys 20 spectrophotometer (Thermo Fisher Scientific). For collagen extraction, samples (~2.5×2.5 mm) were cut from cell-seeded scaffolds and weighed (average wet weight 0.023 g) prior to extraction. Samples were placed in PCR tubes and total collagen was extracted in 100µL of a 0.5M acetic acid and 1 mg/ml pepsin A (Sigma) solution for 16 h on a rocker (Orbitron™; Boekel Scientific, Feasterville, PA) to yield engineered tissue extracts suitable for the Sircol collagen assay.

2.2.8 Scanning Electron Microscopy

Laser microfabricated PGS scaffolds were imaged at a variety of magnifications (e.g., 100x, 300x) using an FEI Quanta 200 environmental scanning electron microscope (ESEM). Specimens were mounted on standard aluminum stubs with conductive tape. Using the low vacuum setting, it was not necessary to sputter coat the specimens. Specimens were imaged under secondary electron mode via the ESEM’s large field gaseous detector (LFD). ESEM parameter values used were a 20 kV accelerating voltage, ~10.5 mm working distance, and 0.6 Torr pressure level.

2.2.9 Phase Contrast Light and Laser Scanning Confocal Microscopy

Cells and cell-seeded scaffolds were routinely monitored using a standard inverted, phase contrast light microscope (TMS; Nikon Instruments, Inc., Melville, NY) under 100x and 200x magnification.
Following 3 weeks cultivation, samples of cell-seeded scaffold were prepared for laser scanning confocal microscopy to image cell nuclei and F-actin by adapting our previously described methods[72]. In brief, samples of cell-seeded scaffold were rinsed in HBSS and fixed overnight at 2-8°C in 10% neutral buffered formalin (Sigma). Fixed samples were rinsed 3x for 5 min each in room temperature HBBS and then extracted in 0.2% (v/v) Triton X-100 (Sigma) in HBSS for 2 h. Following extraction, samples were rinsed 3x for 5 min each in 0.05% (v/v) Triton X-100 in HBSS and then blocked in 1% (w/v) bovine serum albumin (Sigma) and 0.05% (v/v) Triton X-100 in HBSS for 2 h. Following blocking, samples were incubated for 3 h in Alexa Fluor 488-phalloidin (1:40 (v/v) dilution of stock solution in 1% (w/v) bovine serum albumin and 0.05% (v/v) Triton X-100 in HBSS); Invitrogen). The scaffold samples were then rinsed 5x for 5 min each in HBBS, positioned on glass slides, and coverslipped with a drop of Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) to counterstain cell nuclei. Samples were imaged with a x25 water-immersion objective on a FluoView FV1000 laser scanning confocal microscope (Olympus America, Inc., Center Valley, PA), with F-actin and nuclei pseudo-coloured green and blue, respectively. Of note, PGS appeared blue-green in confocal micrographs due to autofluorescence; a method to quench PGS autofluorescence using a solution of 0.3% (w/v) Sudan Black Dye B in 70% ethanol was reported by Jaafar et al. [149].

2.3 RESULTS

2.3.1 Effective Stiffness Measurement

Representative uniaxial tensile stress-strain curves for CIRC and RAD strips of native bovine aortic valve leaflet tissue are presented in Fig.15 B. In particular, the following mean ± standard error mechanical property values were measured (note that E values reported here for native bovine tissues represent peak tangent moduli): $E_{\text{CIRC}} = 1.00 \pm 0.16$ MPa, $UTS_{\text{CIRC}} = 0.251 \pm 0.02$ MPa, and $\varepsilon_{\text{fCIRC}} = 76 \pm 4\%$; $E_{\text{RAD}} = 0.26 \pm 0.03$ MPa, $UTS_{\text{RAD}} = 0.09 \pm 0.01$ MPa, and $\varepsilon_{\text{fRAD}} = 106 \pm 8\%$; anisotropy ratio $E_{\text{CIRC}}/E_{\text{RAD}} = 3.90$. 
PGS pre-polymer was synthesized successfully (i.e., grossly yielded the expected appearance and soft waxy consistency upon cooling to room temperature and was subsequently cured under high vacuum at 160°C for 8, 12, and 16 h and subjected to uniaxial tensile mechanical testing in order to quantitatively compare the polymer synthesized herein to that synthesized previously at MIT[72]. Effective stiffnesses measured in the current study were compared with those obtained previously at MIT [29] in Fig.16, demonstrating a similar trend of increased E with increased curing time at constant temperature. In particular, the following mean ± standard error mechanical property values were measured: E = 1.15 ± 0.06 MPa, UTS = 0.44 ± 0.07 MPa, and εf = 54 ± 7 % (for 8h / 160°C); E = 2.1 ± 0.05 MPa, UTS = 0.64 ± 0.06 MPa, and εf = 34 ± 3 % (for 12h / 160°C); E = 2.47 ± 0.02 MPa, UTS = 0.626 ± 0.04 MPa, and εf = 28 ± 2 % (for 16h / 160°C).

2.3.2 Finite Element Stiffness Prediction

Based on the measured anisotropy ratio of the native leaflet tissues (i.e., E_{CIRC}/E_{RAD} = 3.90), we focused the finite element modeling efforts on simulating scaffolds based on the 2:1 aspect ratio diamond-shaped pore (i.e., the most anisotropic of the diamond-shaped pore geometries available in our custom alumina ceramic mask). Finite element simulations based on the 2:1 aspect ratio diamond-shaped pore, 50 micron wide struts (chosen based on our previous study [29]), and PGS cured for 12 h at 160°C (i.e., having a Young’s Modulus of 2.1 MPa) were conducted (Fig.17) and predicted scaffold effective stiffnesses E_{PD} = 1.10 MPa and E_{XD} = 0.18 MPa; predicted anisotropy ratio E_{PD}/E_{XD} = 6.22.

PGS scaffolds based on this design were microfabricated by 213 nm laser ablation. Representative scanning electron micrographs of the resultant scaffolds comprised of 2:1 aspect ratio diamond-shaped pores and ~50 micron wide struts are presented at 100x and 300x magnifications (Fig. 14 A-B). Representative uniaxial tensile stress-strain plots for separate specimens of these scaffold materials are presented in Fig.16. In particular, the following mean ± standard error mechanical property values were measured for PGS scaffolds comprised of 2:1 aspect ratio diamond-shaped pores (160°C / 12
h curing conditions): $E_{PD} = 0.83 \pm 0.13$ MPa, $UTS_{PD} = 0.4 \pm 0.1$ MPa, and $\varepsilon_{PD} = 62 \pm 14$ %; $E_{XD} = 0.21 \pm 0.03$ MPa, $UTS_{XD} = 0.14 \pm 0.02$ MPa, and $\varepsilon_{XD} = 73 \pm 12$ %; measured anisotropy ratio $E_{PD}/E_{XD} = 3.95$.

Figure 16: Representative uniaxial tensile stress-strain curves for specimens of microfabricated PGS scaffold (2:1 aspect ratio diamond-shaped pores; PGS cured 12 h at 160°C) stretched in the PD and orthogonal XD material directions. Associated effective stiffnesses ($E$), corresponding with the slope of the effectively linear stress-strain curves in the 0-10% strain range, are denoted. Note that $E$ values for the scaffold PD and XD material directions were designed to match the peak tangent moduli of the native bovine aortic valve leaflet tissues in the CIRC and RAD directions, respectively.

2.3.3 Collagen and DNA Quantification Results

To demonstrate their potential to support collagenous engineered tissue formation, scaffold specimens were autoclave-sterilized, pre-conditioned in culture media, and seeded as described above with fetal rat skin fibroblasts previously used to evaluate collagenous tissue formation on similar scaffold structures [82]. Representative phase contrast light micrographs of cell-seeded scaffolds following 3 weeks static in vitro cultivation are presented at 100x (Fig.17 A) and 200x (Fig.17 B) original magnification, demonstrating that the majority of pores appeared to be completely filled with cells and extracellular matrix. A representative laser scanning confocal micrograph (250x original magnification) of a cell-seeded scaffold specimen fluorescently labeled for F-actin and counterstained for cell nuclei is
presented in Fig. 17 C. Biochemical assays for collagen and DNA yielded mean ± standard error concentrations of 736 ± 193 µg/g wet weight (for collagen) and 17 ± 4 µg/g wet weight (for DNA).

Figure 17: Representative phase contrast light micrographs (A;100x; B;200x) of a cell-seeded scaffold specimen following 3 weeks static in vitro cultivation. Scale bars = 200 microns. Representative laser scanning confocal micrograph of a cell-seeded scaffold specimen fluorescently labeled for F-actin (green) and counterstained for cell nuclei (blue) (C; 250x.). Scale bar = 200 microns.

2.4 DISCUSSION

Congenital and acquired cardiovascular diseases collectively represent the underlying cause of 1 in 3 deaths in the US[150], motivating development of new and improved therapies. To be effective, biomaterials in cardiovascular repair or regeneration therapies must at least meet minimum structural-mechanical and biocompatibility criteria. Temporally, cardiovascular prostheses need first withstand acute implantation; ultimately, they need to either recapitulate essential native tissue physiological functions (e.g., the check valve function of a heart valve) or either preserve function or induce functional tissue remodeling (e.g., as mediated by a cardiac support or patch device). Non-viable, non-degradable biomaterials—whether derived from biological tissues (e.g., glutaraldehyde-fixed bovine pericardium) or synthetic from the outset—are typically designed to supersede acute and mid-term implantation mechanical requirements. These conventional biomaterials, however, inherently enter the body limited, either by their suboptimal biocompatibility (e.g., synthetic vascular grafts, mechanical replacement valves) or by their propensity to degenerate in the long-term without the benefit of living cells capable of effecting material repair (e.g., bioprosthetic replacement valves). In hospitable to colonization by
phenotypically quiescent cells and conventional healing, initially sub-symptomatic incompatibilities between the biomaterial and the body gradually manifest in prosthesis-specific combinations of chronic inflammation, calcification, and/or structural-mechanical fatigue. Without re-intervention, such progressive device dysfunctions often culminate in device failure (e.g., restenosis and occlusion due to compliance mismatch and concomitant intimal hyperplasia in conventional small-diameter synthetic vascular grafts). These were and remain some of the clearest and as yet unresolved issues motivating the development and processing of degradable biomaterials into three-dimensional porous scaffolds capable of supporting the cultivation of living cells and the formation of functional engineered cardiovascular tissues.

The current study emphasized scaffolds made of a particularly promising biomaterial, the biodegradable elastomer PGS [145], as well as a relatively deterministic, engineering-based scaffold design and fabrication strategy based on computational finite element simulations [107] and PGS microfabrication by laser ablation [72, 84, 144]. Demonstrating the flexibility of these materials and approaches in cardiovascular tissue repair and regeneration, PGS scaffolds comprised of diamond-shaped pores were designed to match aspects of the mechanics of native semilunar heart valve leaflet tissues. Indeed, in repairing or regenerating load-bearing cardiovascular tissues, mechanical property considerations are of the utmost importance.

*Poly(glycerol Sebacate) (PGS) and Cardiovascular Tissue Mechanical Properties*

Depending on the synthesis equipment, processing conditions (e.g., temperature, vacuum level), device dimensions (i.e., dictating water vapor diffusion distances during curing), and molar ratio of glycerol to sebacic acid utilized during pre-polymer synthesis, a relatively broad range of PGS mechanical properties may be obtained. In conducting research with PGS, it is thus important to recognize that PGS mechanical properties can be highly sensitive to the methods of pre-polymer preparation and final curing. In a similar study, Wang et al. [145] reported a tensile Young’s modulus value of $282 \pm 25$ kPa, tensile strength exceeding 500 kPa, and failure strain of at least $267 \pm 59.4\%$ for PGS (1:1 molar ratio of glycerol to sebacic acid) cured at 120°C for 48 h. Of note, prior to its
development as a biomaterial for tissue engineering, a lipase-degradable PGS pre-polymer was synthesized by reacting glycerol and sebacic acid in a 3:2 molar ratio at 200°C for 120 min and curing at 230°C under nitrogen by Nagata et al. [151]. For comparison, Chen et al. [152] reported Young’s modulus, tensile strength, and failure strains of 220 ± 30 kPa, 390 ± 30 kPa, and 256 ± 26 % for PGS sheets cured at 120°C for 48 h when similarly tested. In Engelmayr et al. [72], a standard curve of PGS effective stiffness (i.e., equivalent to Young’s modulus) versus curing time at a constant curing temperature of 160°C was determined to aid in matching the anisotropic effective stiffnesses of laser microfabricated PGS scaffolds to those of native cardiac muscle. In the current study, a portion of the standard curve of PGS effective stiffness versus curing time at 160°C was repeated as a means to functionally compare the PGS pre-polymer synthesized herein to that synthesized previously (Fig.10). In spite of differences in synthesis and curing equipment, results obtained herein were remarkably similar to those obtained previously [72]. Notably, other recent studies have investigated the application of solid PGS sheets in the context of matching myocardial stiffness in cardiac repair[147, 152], highlighting the potential utility of controlling the mechanical properties of the PGS polymer independent of processing the polymer into a porous scaffold structure.

In the context of porous PGS foam scaffolds, mechanical test data consistently report moduli that are relatively low and effectively isotropic. In particular, PGS foams cured at 120°C exhibited tensile moduli in the range of ~2-9 kPa and failure strains of ~40-80% (estimated from Fig.9 of Crapo et al[87]). A study by Sales et al. on similar PGS foam scaffolds (pores ranging from 75 to 150 µm diameter) reported moduli measured under three-point bending (equivalent to initial moduli measured in tension) of 8.14 ± 0.12 kPa [143]. A third study of PGS foams cured at 150°C for 12, 32, and 36 h reported moduli ranging from 2 to 168 kPa, depending on curing conditions and whether specimens were tested in tension or compression [153].

In the context of PGS membranes and laser-microfabricated PGS scaffolds, mechanical test data presented herein were consistent with those of previous studies [72, 144] and present further evidence that mechanical properties of PGS scaffolds can be targeted and controlled by rational design of pore shape.
Specifically, by selecting data from those studies, the mechanical properties of scaffolds made with similar PGS membrane mechanical properties (i.e., $E \approx 2$ MPa) but different pore shapes can be compared. In the first study, from PGS membranes with moduli of $\sim 2.1$ MPa, scaffolds with accordion-like honeycomb shaped pores and 50 micron wide struts exhibited relatively lower anisotropic effective stiffnesses ($E_{PD} = 0.195$ MPa; $E_{XD} = 0.057$ kPa) than exhibited in the present study by scaffolds with 2:1 aspect ratio diamond-shaped pores and 50 micron wide struts ($E_{PD} = 0.83 \pm 0.13$ MPa; $E_{XD} = 0.21 \pm 0.03$ MPa). By contrast, in the second study using PGS membranes with moduli of $\sim 2.0$ MPa, scaffolds with 2:1 aspect ratio rectangular pores (combined with surface micro-molded linear gratings) and $\sim 50$ micron wide struts exhibited relatively higher anisotropic effective stiffnesses ($E_{PD} = 0.89$ MPa; $E_{XD} = 0.52$ MPa). Collectively, these rough comparisons further highlight the strong influence of pore shape (and associated strut shapes) on microfabricated PGS scaffold mechanical properties. Of note, the influence of pore shape on PGS scaffold mechanical properties was previously demonstrated by comparing square, rectangular, and accordion-like honeycombs. Given the often non-obvious relationships between pore shape and scaffold mechanical properties, the strong influence of pore shape on mechanical properties further emphasizes the need for computational design [107] in targeting specific scaffold mechanical properties.

In the context of PGS scaffolds microfabricated using other methods, Kemppainen and Hollister [94] recently reported tangent moduli (at 10% strain) for PGS and PGS scaffolds microfabricated by a combined solid free form fabrication / molding approach under a variety of glycerol:sebacic acid molar ratios and curing time conditions (all at 150°C). Of note, to the best of the authors’ knowledge, theirs was the only previous study to report mechanical properties of PGS scaffolds measured at 37°C. In particular, they conducted stress relaxation studies under confined compression in 37°C PBS and did not observe any viscoelastic behavior. Subsequently, they conducted unconfined compression tests on cylinders of PGS and measured tangent moduli (at 10% strain) for 1:1 molar ratio PGS ranging from $1730 \pm 920$ kPa (24 h curing at 150°C) to $2820 \pm 490$ kPa (72 h curing at 150°C); these values are consistent with the
tensile modulus value of 2116 ± 100 kPa reported previously by Engelmayr et al. [72] for 1:1 molar ratio PGS cured 16 h at 160°C.

The previous and current studies demonstrate that a broad range of PGS and PGS-based scaffold mechanical properties are achievable by controlling curing time, curing temperature, and scaffold microstructure. Of potential utility in controlling cellular phenotype and differentiation in cardiovascular tissue repair and regeneration applications via substrate stiffness [154, 155] or stiffness gradients [156], PGS and PGS-based scaffold mechanical property values collectively bracket those of native cardiovascular tissues. For example, in the myocardial regeneration context, Bhana et al.[157], Jacot et al. [158], and Engler et al. [159] reported optimal differentiation of neonatal rat cardiomyocytes on polyacrylamide substrates exhibiting stiffnesses ranging from 10 to 50 kPa (i.e., mimicking the stiffnesses of the native tissues from which the cells were isolated). In the case of heart valve tissues, Merryman et al. demonstrated differences in collagen gel remodeling potential [65] and cell stiffness [26] in valvular interstitial cells isolated from pulmonary versus aortic valve leaflets (i.e., from different mechanical environments). Further, Merryman et al. [25] demonstrated that biochemically-induced contraction of valvular interstitial cells can modulate aortic valve leaflet stiffness and Smith et al. demonstrated differences in contractile force generation by valvular interstitial cells isolated from different valves[160][82]. These heart valve-related studies further emphasize that cellular-extracellular matrix (or substrate) mechanical cross-talk is important not only in the myocardial context, but broadly across cardiovascular tissues.

Control of microfabricated PGS scaffold structural-mechanical anisotropy demonstrated herein and previously may also be critically important, both in attempting to mimic native cardiovascular tissues in realistic in vitro models, as well as in preserving their function and/or guiding their regeneration, such as via anisotropic mechanosensing demonstrated by Kurpinskiet al. [161]. For example, in the myocardial context, Fomovsky et al. [162], Gorman et al. [163], and Tanget al. [164] reported compelling evidence that both active [164] and passive restraint [162, 163] of myocardial infarcts may help minimize the progression of pathological remodeling. Using solid PGS sheets, this approach has been developed in
vitro and demonstrated in vivo by Chen et al. [147, 152] and Stuckey et al. [165], respectively. In the vascular grafting context, Lee et al. [166] demonstrated that pulsatile flow conditioning of smooth muscle cell-seeded PGS foam scaffolds (25-32 µm pores) can promote native-like elastogenesis and compliances. Further, Serrano et al. [167] reported significant reduction of intimal hyperplasia by implantation of a perivascular graft based on a nitric oxide (NO) secreting poly(diol-co-citrate) bioresorbable elastomer developed by Zhao et al. [168]. Indeed, poly(diol-co-citrates) developed by Yang et al. [169] represent another promising class of bioresorbable elastomers.

In the current study, while cytoskeletal stress fiber organization was evident within the fibroblasts filling and spanning the diamond-shaped pores of the cell seeded PGS scaffolds by F-actin staining (Fig. 17 C), cell and collagen fiber alignment were not explicitly quantified (as for microfabricated PGS scaffolds using a Fast Fourier Transform (FFT)-based method [72]). Because cells were seeded at a high density (i.e., ~10x10^6 cells/cm²) resulting in rapid aggregation and filling of pores, the growth front-mediated orientation effect (preferential along the pore long axis) observed previously [82] in similarly shaped pores seeded with the same fibroblasts at a low density (i.e., ~7.5x10^5 cells/cm²) was not explicitly expected.

Importantly, the geometrically well-defined pore shapes of microfabricated PGS scaffolds are expected to facilitate finite element simulations of bioreactor-mediated mechanical loading, such as simulated previously for mesenchymal stem cell-seeded collagen gels by Pfeiler et al. [170].
3 THEORETICAL AND FINITE ELEMENT ANALYSIS OF MICROFABRICATED POLY
(GLYCEROL SEBACATE) SCAFFOLDS

In Chapter 2, we confirmed the capability of PGS scaffolds to promote collagenous tissue formation while matching the anisotropic mechanical behavior of native bovine heart valve leaflets [45]. In addition, previous studies demonstrated the important role of tissue architecture (cell and collagen arrangement) in the mechanical properties of TEHV that affect the heart valve leaflet functionality [51, 143]. In the context of microfabricated scaffolds, it has been shown previously that pore geometry influences the scaffold’s mechanical characteristics and anisotropy and could direct the cellular arrangements and guide the tissue formation in the TEHV [82]. Thus, for better understanding the scaffold’s structural geometric role on its stiffness, mathematical modeling and FE analyses in the current chapter were developed to evaluate and optimize the mechanical properties of designed microfabricated PGS scaffold (comprised of diamond pores) prior to and after cell seeding the constructs. This helps to optimize the scaffold’s design in further fabrication to create a construct that resembles native tissue better. In specific, 2D and 3D FE model of scaffolds and cell seeded composite were generated to predict the effective stiffness of the elastomeric scaffold and engineered tissue as a function of pore geometry and tissue content, respectively. At first, the predicted effective stiffness of scaffolds was compared with experimental data, obtained from uniaxial mechanical tester, to validate FE analysis. The stiffness obtained from FE analysis of representative scaffolds for two orthogonal material directions (PD&XD) were verified with the experimental data. The findings indicated that the microfabricated PGS mechanical characteristic follows, to some extent, a rule-of-mixtures (corrected for angle) behavior but not precisely in both orthogonal directions. Furthermore, FE analysis was used to develop a correlation between the scaffold’s pore geometry /strut width and the scaffold’s stiffness to determine an appropriate structural design that resembles native leaflet’s stiffness and anisotropy. Results showed that the scaffold increased in anisotropy as the angle of the diamond shaped pore diverged from 90°. The scaffolds volume fraction decreased from 30° to 90° diamond angle. Similar trend was obtained while changing the strut width in the
scaffolds. Higher volume fraction and lower anisotropy were associated with scaffolds with larger strut width. Finally, FE analysis of cell seeded scaffolds predicted the effective stiffness of a composite as a function of scaffolds and tissue (formed inside the diamond pores) stiffness.

3.1 INTRODUCTION

The scaffold, used for creating functional TEHV, should mimic native leaflet anisotropic mechanical characteristics at the time of implantation and during the remodeling process. The scaffolds support the cell growth, proliferation, and ECM formation while it slowly degrades, with the aim that collagen fibers replace the scaffolding material. Synthetic scaffolds, fabricated with a spinning method, have shown to have limitations in terms of producing uniform pore [50]. In those scaffolds usually it is hard to control the pore sizes and arrangement in the scaffolds. In addition, it is difficult to see the two orthogonal directions clearly (PD and XD) (Fig.5) of Engelmary et al. [50]) due to the nonuniformity of the structure and randomly distributed of the fibers which leads to a non-anisotropic characteristic for those non-woven [50] or electrospinning scaffolds [57]. For the same reason, controlling the mechanical characteristics of those scaffolds showed to be difficult. Microfabricated scaffolds on the other hand, showed to be more consistent in mechanical characteristic and porosity since these scaffolds consisted of uniform pore density and pore geometry. As investigated previously, Engelmayr et al. developed an accordion-like honeycomb PGS scaffold toward replicating aspects of cardiac structural mechanics, combining the precision of microfabrication with cardiac bio-mimicry [82]. Considering this and elastic characteristics of PGS material, we believed that it is feasible to control the scaffold’s mechanical characteristic and anisotropy by manipulating fabrication design and PGS curing condition (curing time and temperature under vacuum). As mentioned previously, PGS scaffolds were computationally designed and laser microfabricated to match the anisotropy and the peak tangent modulus for bovine native heart valve leaflets [45]. Here, we adopted the PGS scaffolds with similar design, then fabricated with micromolding technique, and simulated the structure and mechanics of those constructs. This study was carried out in an effort to understand the mechanical characteristics of microfabricated PGS scaffold with
diamond shaped pores by quantifying the effects of pore geometry on the scaffold’s anisotropy. The computational models could be modified to develop the appropriate pore geometry that result in scaffold stiffness and anisotropy similar to the native valve leaflet tissue characteristics. In addition, using FE models enabled us to gain a better understanding of tissue content formed inside the diamond pores.

3.1.1 Previous Theoretical and Finite Element Modeling of TEHV

Optimizing the mechanical properties of scaffolds is becoming more feasible with the help of mathematical and computational modeling such as FE analysis. In 2005, Engelmayr et al. developed a composite beam model that accounts for the effects of nonwoven scaffolds ECM and variations in collagen concentration [51]. The model was then used to predict the effective stiffness in the ECM of TEHV samples incubated under static and cyclic flexure conditions. Here, a similar mathematical model was also used to predict PGS scaffold’s stiffness, containing uniform diamond shaped pores. Many published studies used mathematical modeling and FE analysis in computational models to study normal, pathological, and prosthetic valves [32]. Some FE models were developed to evaluate the mechanical interaction of native valve leaflets and the physiological environment [25, 101, 102, 171-175]. However, few studies, based on computational or theoretical modeling, were devoted to predict the strength and stiffness of biomaterials used in heart valve tissue engineering [50, 51, 107, 176, 177]. Regarding nonwoven PLLA/PGA scaffolds, most of the mathematical models, associated with the structural mechanics of nonwoven textiles, began in earnest with Backer and Petterson [176] and application of the fiber network theory proposed by Cox [178]. Later, investigators refined this theory, quantifying and modeling for fiber orientation [179] on the mechanical response of the fabric. Toward predicting these changes, Engelmayr et al. [50] developed a structural model for stiffness (E) of a nonwoven scaffold that accounted for the number and orientation of the fibers in the scaffold. He also predicted the spring-like effective stiffness of the sinusoidally curved fibers. In a recent study done by Jean et al. [107]finite element 2-D simulations on a homogeneous PGS scaffold were used to predict the anisotropic effective stiffness of PGS scaffold with accordion-like honeycomb (ALH) shaped pores with the aim of matching
the anisotropic mechanical properties of native cardiac muscle. FE predicted ALH scaffolds with 50mm wide PGS struts to be maximally anisotropic.

In the current study, microfabricated PGS scaffolds comprised of diamond shape pores were generated using a micromolding technique [46]. Tensile mechanical testing, on bare PGS scaffolds and cell seeded scaffolds, was conducted to define the mechanical properties of those samples. 2D and 3D representative models of the constructs were generated in two directions; (PD) and (XD), and FE simulations were used to predict the anisotropic effective stiffness of the designed PGS scaffolds. The elastic moduli and associated anisotropy obtained by FE simulations were compared to the effective stiffnesses measured in experiments (by a uniaxial-mechanical tester) for PGS scaffolds cured 13h/160°C, reported in [46]. Results were also compared to the data derived from theoretical analysis, rule-of-mixtures (corrected for angle). FE analyses were conducted in commercially available simulation software CATIA (V5, R14), COMSOL and periodic tessellation SIMULINK (described in the Chapter 2). Following this step, the individual pore geometry parameters were changed in simulations to find a correlation between the scaffold's stiffness and pore geometry variables. This assists to obtain the most appropriate microfabrication design which results in the scaffold with mechanical properties close to the native tissue stiffness and anisotropy. Finally, based on the scaffold’s stiffness and tissue formation in the composite, the cell-seeded composite overall stiffness was defined.

3.2 MATERIALS AND METHODS

For the first part of the study, a theoretical calculation of scaffold stiffness (using rule of mixtures corrected for the angle [55]) was adopted and results were compared to experimental findings and FE analysis predictions. In addition, FE analysis was used to associate the scaffolds pore geometries to the stiffness of the microfabricated PGS constructs. Finally, a composite cell seeded scaffold model was made using FE to predict the stiffness of the TEHV stiffness versus tissue stiffness formed within the scaffold’s pores.
3.2.1 Experimental Results for Microfabricated PGS Scaffold and Cell Seeded Scaffolds

The experimental methods regarding PGS synthesis, scaffold microfabrication, cell seeding and cultivation process were discussed in detail in Chapter 2. In brief, PGS polymer was synthesized by a method adapted from Wang et al. [145], reacting glycerol and sebacic acid (1:1 molar ratio, Sigma) under heat (120°C) and N₂ for 24hrs, followed by 24hrs high vacuum (~15 mTorr) to yield a viscous pre-polymer. PGS was fabricated by a micro-molding process on ceramic molds (ultra-high temperature machinable glass-mica ceramic sheet (0.5" thick, 2"×2", McMaster)) using a dicing cutter machine (Kulicke & Sofia, Indus. Inc., 1998). Pre-polymer melted on a mold and was cured in a vacuum oven (160°C and 12hrs; PGS Young’s modulus of 1.77 MPa) yielding scaffolds comprised of approx. 2:1 aspect ratio of diamond shape pores and a 65 micron strut width. The procedure has been described in Chapter 4 in detail.

Cell seeding techniques have been described in Chapter 2 in detail. Scaffolds were seeded with rat skin fibroblast for 4 weeks. PGS scaffolds and the cell-seeded scaffolds were prepared and subjected to uniaxial tensile testing (LLOYD) as described previously.

3.2.2 Rule of Mixture, Corrected for Angle based on Composite Material Model

The simple composite material model (i.e., rule of mixture corrected for angle) was used to estimate the scaffold’s stiffness by knowing the volume fraction of the PGS material in scaffolds. In this case, the scaffold’s struts were considered fibers that were aligned in two directions with respect to y direction (27.9° and -27.9°) (angle α; Fig.18) and the void stiffness was set to zero in the general equations. This angle of 27.9° was also calculated from the measurements of scaffold dimensions obtained from ESEM images of scaffolds, created in the experimental phase.
Figure 18: (A) Schematic model of microfabricated scaffold which depicts the diamond pores geometry variables, including strut width $w$, diamond angle $\alpha$, diamond length $l$ and diamond long axis $d$, (B) ESEM micrograph of microfabricated PGS scaffolds were used to (C) generate 3D geometry model for FE analysis.

The general equation of effective stiffness of a composite material based on scaffold fiber alignment and composite volume fraction is:

\[
(2-1) \quad E = n_0 E_f \nu_f + E_m \left(1 - \nu_f\right)
\]

\[
(2-2) \quad n_0 = \sum \alpha_i \cos^4 \theta_i
\]

Where $E$ is the effective stiffness of the composite material (cell seeded scaffolds), $E_f$ and $E_m$ are the scaffold's fiber (in this case scaffold's struts) and tissue (matrix) effective stiffnesses respectively (note for scaffolds, this part would be considered void), $\nu_f$ is the scaffold volume fraction and $n_0$ is a corrective factor for angle of polymer fibers with respect to the loading axis. In this case, $n_0$ is defined by the angle of struts with respect to the loading axis, as defined by the geometry of the diamond shaped pores (Fig.18 A). For a composite comprised of unidirectionally oriented structural members, $n_0 = 1$, and for a scaffold comprised of randomly oriented fibers it is 0.375. For our geometry $\theta_i = 27.9^\circ$, this led to:

\[
(2-3) \quad n_0 = 0.5 \cos^4(27.9^\circ) + 0.5 \cos^4(-27.9^\circ) = 0.61
\]
By reverse engineering and defining the volume fraction of the scaffolds based on the pore geometry, we could define the effective stiffness of the cell seeded scaffolds and relate the data to experimental results.

3.2.3 Description of Geometry Model and Finite Element Simulation

3.2.3.1 3D simulation of scaffolds and cell seeded scaffolds

To verify the FE analysis results, models with 4mm width, 0.25mm thickness and 7mm length (in both PD and XD directions) were generated based on the experimental samples’ sizes (Fig. 18 C) to predict the PGS scaffolds elastic stiffness. The precise 3D models were simulated based on scaffold’s dimensions obtained from ESEM images of the microfabricated scaffolds (Fig. 18 B). Different sets of scaffolds (PD and XD models) with variable geometries (i.e., diamond angle α = 30, 45, 65, 90) and strut width (35, 50, 60, 80 µm) was generated once with constant diamond length (l) of 200 µm and once with constant long axis of diamond (d: 240 µm) to predict the scaffolds stiffness as a function of pore geometry and scaffold strut widths. Parameters defined mostly in accordance with a feasible design that could be obtained in fabrication procedures. Finally, similar to the first step, light microscopic images of the cell seeded samples were used to design a model of the cell seeded scaffold (Fig. 19 A-C) and homogenous material was considered inside the diamonds pore, as tissue, for simplicity. As shown in the confocal images (Fig. 19 B), following 4 weeks cells cultivation on the fabricated scaffolds, tissue formed inside the diamond pores and the effective mechanical behavior of the overall composite was computed with FE analysis based on scaffold material stiffness and tissue composition.
Figure 19: (A) ESEM image of cell seeded scaffolds comprises of tissue formation inside the diamond pore geometries. (B) The confocal micrograph of cell seeded scaffolds presented the stained F-actin proteins in cellular organization. (C) Geometrical model of cell seeded scaffolds were generated based on experimental images of cell seeded scaffolds.

3.2.3.2 Boundary conditions and FE analysis of scaffolds stiffness

The PGS structural elements were assumed to exhibit isotropic linear elastic behavior based on uniaxial mechanical tester results [45, 46]. For the PGS material, Young’s modulus was assigned 1.77 MPa based on the uniaxial tensile testing (for PGS membranes cured 12h /160°C) and soaked in 60°C distilled water over night. Poisson’s ratio equal to 0.49 was also assumed for the scaffold structure. All parameters used in FE analyses have been presented in Table 1. Boundary conditions, to predict the scaffold’s stiffness, were defined on the models based on the experimental set up. The models were fixed from one edge and stretched through the length from the other edge, according to uniaxial tensile loading tests. Loads ranging from 0 to 0.2 Pa were applied when testing the models (Fig.20). For individual applied stress or force, the corresponding strain values were measured and the linear stress-strain curve was plotted to measure the PGS scaffold’s stiffness (in the two orthogonal PD and XD directions). To quantify the influence of fabrication design dimensions (pore geometry and strut width), similar simulations and boundary conditions were applied to predict the scaffold’s effective stiffnesses and anisotropy (E_{PD}, E_{XD} and V-PGS) were predicted for a range of diamond pore angles (α) and strut widths (w) with constant diamond length or long axis). To compute the effective stiffness, the computational finite element equations were used to obtain the stress and strain values.
Table 1: Parameters used in the FE model for the scaffold geometry and material

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scaffold Geometry</strong></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>7mm</td>
</tr>
<tr>
<td>Width</td>
<td>4mm</td>
</tr>
<tr>
<td>Height</td>
<td>0.25mm</td>
</tr>
<tr>
<td><strong>Scaffold Material Properties</strong></td>
<td></td>
</tr>
<tr>
<td>Young's Modulus</td>
<td>1.77MPa</td>
</tr>
<tr>
<td>Poisson's Ratio</td>
<td>0.49</td>
</tr>
<tr>
<td>Density</td>
<td>1235</td>
</tr>
</tbody>
</table>

Figure 20: Diagram of the applied boundary condition on the (A) PD and (B) XD models includes a fixed wall on one edge and applied force on the other edge resembling the uniaxial mechanical testing procedure.

Following FE analysis verification and obtaining correlation between the scaffold’s structure and mechanical characteristics, the cell seeded model was developed based on light microscopy images of a cell-seeded scaffold (Fig.19). The tissue formation within the void spaces in the model was assumed homogenous and isotropic material. The effective stiffness of cell seeded composite was predicted versus various Young’s moduli of the tissue content in the diamond pores (ranging from 30 kPa to 120 kPa) and scaffolds’ Young’s moduli (ranging from 500 kPa to 2 MPa). Then the composite stiffness was plotted based on tissue content inside the scaffold’s pores and stiffness. Of note, the initial linear trend of stress-strain curve (for cell seeded scaffolds) observed in experiments (details in Chapter 4) suggested that the viscoelastic behavior of ECM is regardless due to small amount of protein content in the engineered tissue.

56
(during the short period of \textit{in vitro} cultivation). Therefore considering the tissue content inside the diamond pores, a homogenous elastic material is not far from reality.

3.3 RESULTS

The first attempt was to compare the predicted stiffness of PGS scaffold with the experimental and theoretical (rule of mixtures) data. The scaffold’s pore geometry was then modified to develop a correlation between structural parameters, including angle ($\alpha$), strut width ($w$), and scaffold stiffness. The FE models were used to calculate the volume fraction and the anisotropy ratios of scaffolds as a function of pore geometry. Finally, a 3D graph of overall stiffness of the cell seeded composite was plotted based on the stiffness of tissue (formed inside the diamond pores) and scaffold’s stiffness.

3.3.1 Theoretical Model and FE Effective Stiffness Prediction Vs. Experimental Data

The stiffness measured from the FE analysis, rules of mixture theory and experiments are summarized in Table 2 (results have been presented in the two orthogonal directions). In particular, the following mean $\pm$ standard error mechanical property values were measured in experiments. Of note, the ultimate tensile strength (UTS) and strain to failure (ef) can be only obtained from experimental data. The results for FE analysis are close to the experimental data (0.74 vs 0.87 $\pm$ 0.065 and 0.124 vs 0.128 $\pm$ 0.098 MPa). Representative uniaxial tensile stress-strain curves obtained in experiments and computational modeling in the two orthogonal directions (PD and XD), are compared in (Fig.21 A). We obtained a linear trend of stress-strain curves for PGS scaffolds in experiments due to its elastic characteristics. FE model also simulated a similar trend of stress-strain based on applied forces and measured strain values since the material was considered homogenously elastic. The slope within this stress-strain curve indicates the Young’s modulus or effective stiffness of the scaffold. Between the FE and experimental results, the Young’s moduli were quite comparable (with ~9% difference for PD and ~3% difference for XD). Note that the XD oriented scaffold samples had a better fit between the FE and experimental results than the PD oriented scaffolds. However, theoretical measurements predicted the scaffolds stiffness with
a deviation of 19% and 26% in PD and XD directions, respectively. Regarding the pore orientation, PD results showed a lower stiffness within the scaffold when compared to XD results.

Table 2: Stiffness of PGS scaffold for FE and theoretical results and stiffness, ultimate tensile strength, and strain to failure for experimental results for both the PD and XD pore orientations

<table>
<thead>
<tr>
<th></th>
<th>FEM E (MPa)</th>
<th>Theoretical E (MPa)</th>
<th>Experimental E (MPa)</th>
<th>UTS</th>
<th>εf</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>0.740</td>
<td>0.600</td>
<td>0.876 ± 0.065</td>
<td>0.250 ± 0.059</td>
<td>0.422 ± 0.032</td>
</tr>
<tr>
<td>XD</td>
<td>0.124</td>
<td>0.047</td>
<td>0.128 ± 0.098</td>
<td>0.090 ± 0.019</td>
<td>0.474 ± 0.086</td>
</tr>
</tbody>
</table>

We applied 0.250 MPa stress (corresponding with UTS values obtained from the experimental data) on the boundary of the PGS scaffold in PD direction and the maximum stress values distributed on the scaffold’s struts was obtained (0.31 MPa). This value is similar and corresponded with UTS of the PGS polymer sheet (0.371 ± 0.075 MPa) (cured for the same amount of time as scaffolds curing conditions (13h/160°C)). However, comparing the local maximum stress (0.31 MPa) with PGS scaffolds UTS (0.250 ± 0.059) confirmed that the presence of the micro-pores in the scaffolds decreased its toughness and strength, compared to the PGS membrane strength, due to the void spaces. The strain to failure of the scaffolds, on the other hand, is higher than PGS membrane strain to failure, due to lower stiffness of the microfabricated scaffolds and presence of diamond pores (0.323 ± 0.012 vs 0.422 ± 0.032).

3.3.1.1 Stress and Strain Distribution Contour

Stress and strain distribution on the scaffold struts and scaffolds’ deformations have been presented for both PD and XD models in Fig.21 B-E. As shown, the maximal stress and minimal strain in both cases was seen in the struts close to diamonds edges. Scaffolds were the most compliant at the connection points and produced, therefore, greatest stresses and strains are distributed at the stiffer regions of the struts which are the connection points. Therefore, local maximum stress and minimum strain values were obtained in the diamonds’ edges.
Figure 21: (A) Stress-strain curve for the best fit of experimental data and FE results for both PD and XD pore orientations. The plot shows linear trend lines which fit the data well. (B, D) strain and stress and (C, E) distributions for PD and XD scaffold stretched using a 0.1MPa load. (B, D) shows a zoomed-in image (center of the scaffold) of the strain, showing the deformation that is occurring in PD and XD directions. (C, E) Similarly, the overall von Mises shows a zoomed-in image (center of the scaffold) of the stress. Note that the deformation shows that the scaffolds displace in both the x and y directions. Maximum stress values were observed on diamonds’ edges.
3.3.2 **Representative Volume Element (RVE) Determined for Scaffold**

The correlation between the number of diamond pores and the scaffold’s elastic stiffness were determined and depicted in Fig.22. The designed scaffold’s stiffness was measured as a function of the unit cells (diamond pores) number (for the two orthogonal directions; PD & XD). The predicted stiffness values approached a steady level, comparable to the experimental data (Table 2), for scaffolds containing the minimum of 61 diamond pores in PD direction and 81 diamond pores in XD directions in total. Therefore, the minimum dimension of the scaffold which leads to the real stiffness value should be considered greater than about 5mm by 5mm and with about 100 diamond pores arrangement in the design. (Diamond pore geometry was set as experimental samples with 27.9° angle and 65 µm strut width and about 240 µm long axis of diamond).

Figure 22: **Scaffolds Stiffnesses** in the orthogonal PD and XD directions as a function of the unit cells numbers in the geometrical FE model, obtained FE analysis for 13 h of curing at 160 °C.
3.3.3 Scaffold Effective Stiffness Vs. Pore Geometry and Strut Width

The scaffolds were designed with different diamond pore geometries and the relative effective stiffness were measured to obtain the anisotropy ratios \( r = \frac{E_{PD}}{E_{XD}} \) were illustrated as a function of diamond angles, \( \alpha \) and strut width \( w \) (once with a constant diamond length \( l \) of 200 µm and once with constant diamond long axis of 240µm) (Fig.23 A-B). Scaffold consisting of 90° angle diamonds showed an isotropic elastic behavior with \( r=1 \) as a homogenous material. The scaffold with designed \( w/l=0.25 \) and angle of 30° was found to have a highest anisotropy ratio \( (r=45) \) in the model with constant \( l=200\mu m \). The scaffold with designed \( w/d=0.15 \) and angle of around 30° was found to have the maximum \( r=22 \) for the scaffold. Similar measurements lead to volume fraction of PGS \( (V_{PGS}) \) for designed scaffolds as a function of pore geometries. Volume fraction of PGS decreased with increasing diamond angle, \( \alpha \), as expected. Scaffolds with bigger diamond pores (larger \( \alpha \) angle) include larger void areas leading to smaller \( V_{PGS} \). Of note, the results will be similar passing the 90° angle as the PD samples will be determined as XD samples.
Figure 23: Correlation between the ratio \( r: \frac{E_{PD}}{E_{XD}} \) to angle \( \alpha \) (A) showing a decrease in anisotropy as the angle passes about 30° and approached 90° for the model with constant length (l=200µm) and (B) constant diamond long axis (l=240µm). The curves are the best fit trends for the data and are solely meant to visually show the trend in the data. (C-D) Volume fractions of PD oriented scaffold as strut width changes for the model with constant length and constant diamond long axis respectively.

The maximum stress and strain values distributed on the scaffolds were obtained with FE analysis in scaffolds with different strut widths (constant angle and diamond length (\( \alpha=65^0 \) and l=200µm) and data were compared (Fig.24 A-B). Models were stretched with similar load magnitude, and as expected, scaffolds with smaller strut with hold the larger values of stress (0.49 MPa max. stress in 30 µm strut width versus 0.29 MPa max. stress in 80 µm strut width). They also deformed with lesser amount of strain (0.617 strain in 30 µm strut width versus 0.343 strain in 80 µm strut width). The anisotropy ratio of scaffold (initially made in phase 1), did not change with the change of scaffold material stiffness (E
Young modulus) because there is a linear relation between scaffold stiffness and material characteristics (value remain at r:6).

Figure 24: (A) Maximum local stress and (B) average global strain values obtained from FE analysis versus scaffold’s strut width. Scaffolds with smaller strut width demonstrated larger strain distribution on the strut width and lower stress magnitude spread on the strut widths.

3.3.4 Predicting Tissue Formation Stiffness based on FE Analysis

Based on the results, there is 300% higher average value of stress distribution on the struts width compare with average stress distribution on tissues inside the diamond pores (Fig.25 A). This is due to the higher stiffness of the scaffolds compared to the tissue stiffness formed inside (in the initial run, the scaffolds stiffness was considered 1.44 MPa, obtained from experiments and tissue stiffness was considered 140KPa based on cell stiffness). To measure the cell seeded composite stiffness versus those stiffnesses, the PGS stiffness has been varied between 0.5-2.5 MPa based on uniaxial mechanical tester results obtained for cured PGS membrane at different time and temperature conditions and tissue stiffness was adjusted between 0.5-2.5MPa based on cell stiffness and ECM stiffness [72]. Of note, tissue content was considered a linear homogenous material. The 3D graph presented in Fig.25 B is the composite scaffolds stiffness as a function of scaffolds and tissue stiffnesses. Using this graph, we can determine the stiffness of tissue formed inside the diamond pores based on seeded and unseeded scaffolds’ stiffnesses from experimental measurement with the uniaxial testing device.
Figure 25: (A) FE results of stress distributions within cell seeded scaffold model and the overall deformation. Due to higher stiffness of the scaffolds compared to tissue stiffness stress values are considerably higher on scaffolds struts than the stress distributed on the tissues in the pores. (B) The 3D graph depicts the effective stiffness of cell seeded composite vs scaffolds and tissue stiffnesses.

3.4 DISCUSSION

Heart valves are complex structures, anatomically and physiologically, due to their cellular and ECM architectural arrangement. Elastic microfabricated PGS scaffolds have been used to design a biomimetic construct that matched the native tissue leaflets anisotropy and mechanical characteristics [18, 45, 46]. Previously Hollister et al. [180], presented the advantages of FE analysis on microfabricated scaffolds. The primary aim was to develop an accurate geometrical model with FE analysis to investigate the scaffold’s structural and mechanical parameters, contribution toward cell seeded TEHV mechanical characteristics. Scaffold’s geometrical and mechanical characteristics play an important role on its functionality [181] especially TEHV’s anisotropy. Designed scaffolds exhibited anisotropic mechanical characteristics due to the structure of fabricated diamond patterns. Evaluation of various microstructure and pore geometries in scaffolds prior to the actual fabrication process is the main achievement of FE analysis in this study. Previously, the importance potential role of cell and collagen orientation on mechanical properties of TEHV on leaflets functionality was discussed through literatures [11, 51, 182].
Thus, the scaffold’s mechanical properties and organization of collagen and cells could be the most important factor to be controlled in heart valve tissue engineering.

Through this study, we were able to model the structural mechanics of a PGS scaffold with uniform diamond shaped pores. The model was compared to and supported by experimental results. Thus, the present model was able to predict the elastic behavior of microfabricated PGS scaffolds in both orthogonal directions. For both orientations, the theoretical model was not very comparable to both the FE and experimental results, meaning that a more accurate theoretical model is needed for this specific PGS scaffold construct (Table 2). This could be due to modeling the strut widths as fiber structures and ignoring the interconnection between the struts present in the PGS scaffold structure. In the actual rules of mixture, the fibers are considered to be staged on each other with no fusion or connection points. The slight difference between the FE analysis and experiments on the other hand, can be explained by the fact that the scaffolds bulk are not an elastic homogenous material, consisting of evident microspores which simulating those effects is beyond the simplification of this study and could be investigate in future studies. Also, it has been observed that the pore geometry are not precisely uniform through the thickness of the scaffolds fabricated by micromolding procedure. Regarding the two PD and XD directions, it is evident in the theoretical, FE, and experimental results that the PD pore orientation produced a higher stiffness within the scaffold when compared to the XD pore orientation. This result indicates that the larger angle $\alpha$ within the XD oriented pores allows for a greater displacement within the material as the load is applied. The difference in the stiffnesses with these two directions indicates an overall anisotropic behavior of the scaffold. The material, however, can be considered isotropic when only considering the x-direction. As described in results, the maximal stress was observed (for both PD & XD) on the edge of diamonds on scaffolds’ strut with and maximal strain in both cases (PD & XD) was seen in the middle of the strut. This result agreed with what was expected since the scaffolds were the most compliant at the connection points and produced the greatest stresses and strains at the stiffer strut region. Considering that, it was also observed in the experiments that scaffolds were ruptured from the joint points. The
deformation occurring as the scaffold stretches can also be seen in Fig. 21 B-E. As seen in these zoomed-in images of the center of the scaffold, deformation occurs in both the x and y directions.

To better understand the failure properties of microfabricated scaffolds, the average global strain distribution and maximum local strain values on scaffolds struts were compared to strain to failure for scaffold and PGS sheet (cured with in the same time period). Similar comparison presented for the maximum local stress distribution on scaffold’s struts and values were compared to scaffold’s and PGS sheet’s UTS. The results indicate that the strain and stress distributed on the scaffolds struts are similar to the failure properties of PGS membrane measured experimentally. Following validation of geometry simulation via comparison between predicted stiffnesses and experimental data, the RVE contained about 80 diamonds indicated that the effective stiffness of the scaffolds, containing lesser number of 81 unit cells, is very much dependent on the number of the pores fabricated on the scaffolds (Fig. 22). The experimental sample’s size, tested for uniaxial tension, was reported as (4mm×7mm), which comprise of enough number of unit cells that lead to a steady result of stiffness. The geometric and architectural cues critical for guiding cell alignment and orientation are most often studied using various 2D or so-called 2.5D models, where lithographic and other microfabrication methods have been used to systematically evaluate parameters such as feature shape and size, which influence cell alignment.

The heart valve leaflet’s functionality is dictated by native tissue mechanical characteristics and most importantly, its anisotropy [9, 11]. This study was also carried out in an effort to understand the structural mechanics of designed a scaffold with diamond shaped pores by quantifying the effects of pore geometry on the stiffness of the scaffold. The computational models could later be modified and used to develop a design for microfabrication that produces scaffold stiffness similar to native valve leaflet characteristics. The scaffold’s stiffness anisotropy ratio, r, was calculated as a function of pore geometry and strut widths. These values are correlated with the PGS volume fraction. Though lower values of diamond angle resulted in higher anisotropy ratios which is closer to native tissue’s stiffness anisotropy (~15) [46], those scaffolds are weaker and would rupture in a lower strain to failure due to high stress.
values (0.5 MPa), distributed on the scaffold’s strut widths. As seen in Fig.23 C-D, volume fraction was dependent on the change of strut width and angle and increased as strut width increased and decreased as diamond angle increased. This is evident as void volumes increased in larger diamond pores. The increase in pore volume decreased the volume of the PGS scaffold; however, the reduction in the strut width had the opposite effect.

By modifying the geometry of the diamond pores, it was determined that scaffold anisotropy is dictated by the angle α and strut widths. The ratios between two orthogonal direction stiffnesses (E_PD/E_XD) represent the anisotropic behavior of the scaffold with respect to pore geometry. As the scaffold becomes more isotropic (α=90°), the expected ratio of E_PD/E_XD approaches 1. As seen in Fig.23 A-B, the scaffold anisotropy increased as the angle α decreased (the highest ratio of E_PD/E_XD being seen at the angle of 30°). Also changing the strut width had less of an effect on the anisotropy of the material as the angle approached 90°. Strut width tended to affect the material stiffness more when the angle was reduced. Overall, the anisotropy of the material was dictated by the angle of the diamond pore (α) when the volume fraction was only affected by a change in strut width.

Having a scaffold that can deform (comprising larger strain to failure and local maximum strain on the struts) and stand high amount of stress (maximum local stress) is important for designing a microfabricated scaffold with improved mechanical characteristics. Therefore, while the lower strut width demonstrated higher anisotropy ratio (closer to native tissue values anisotropy), higher stress and strain were distributed on those struts. This confirmed the fact that scaffolds with thicker strut widths resulted in an improved failure properties (Fig.24 A-B). The scaffold’s stiffness and PGS stiffness are linearly related. Therefore, the curing conditions (time and temperature) can be adjusted according to the PGS polymer stiffness to resemble the native tissue characteristics accordingly.

Finally, a cell-seeded model was made to predict the stiffness of the tissue formation occurring within the composite. PGS degrades over time by surface hydrolysis process [183, 184]. Therefore, it has been observed through the experimental analysis that the scaffold’s stiffness diminished over the time due
to loss of PGS volume fraction. Previously, reduced values of the effective stiffness and anisotropy of the accordion like honey comb scaffolds were observed following 4 weeks of cell culture (i.e., EPD = 0.032±0.002 MPa, EXD = 0.019±0.004 MPa, r=1.9±0.3) that could be due to the combination of scaffolds degradation and tissue formation [72]. In order to better understand the degradation process and tissue formation in the composite, the cell seeded scaffold was simulated and evaluated for mechanical characteristics based on the PGS elasticity and tissue formation. This model allows us to predict the stiffness of tissue formation inside the pores based on the experimental data of the PGS scaffolds and cell seeded composite stiffnesses. Different values were set for scaffold’s softness in the simulation is correlated with the degradation of PGS material. Knowing the overall composite stiffness from uniaxial testing of seeded scaffolds and measuring unseeded scaffolds stiffness, that has been soaked equal with tissue culture time, (uniaxial testing of the control unseeded scaffolds following degradation during culture time) will determine the tissue elasticity inside the pores through the graphical analysis shown in Fig.25 B. Tissue stiffness varies from cell stiffness values to collagen fiber and ECM network stiffness reported in literature (collagen and VICs stiffness). Considering that and obtaining the tissue elasticity from the simulation helped us to gain an indication of tissue evolvement through the cultivation time [185]. The stress and strain distributed on scaffold’s strut were observed to be considerably higher than those on tissues inside the diamond pores (based on experimental data obtained for cell seeded scaffold’s stiffness ~1.6MPa and unseeded PGS scaffolds stiffness ~1.44MPa). This is due to the fact that scaffold’s stiffness is significantly higher than tissue elasticity and therefore, the scaffold is the main load barring element in the composite.

3.4.1 Limitation

Within the FE modeling, there are various limitations. Overall, this model considered a uniform scaffold which has uniform porosity and pore geometry. Within the experimental phase, this was likely not the case as scaffold pore geometry and porosity can change based on the microfabrication design. Within the cell-seeded scaffold model, the tissue in the pores was also assumed to be uniform which
exhibit isotropic linear elastic behavior similar to the PGS structure. Although, this assumption is far from reality due to viscoelastic behavior of ECM [32, 186], we have seen mostly a linear trend of stress-strain (in the initial 20% strain) for the cell seeded scaffolds. Therefore, we believe, since the tissue formed inside the pores is not as mature as their native structure, we can disregard the viscoelastic characteristics of ECM fibers. Also, the dependent variables of cell orientation and collagen fiber arrangement were ignored for the simplicity and the tissue formed inside the pores was considered homogeneous and the stiffness was assessed based on the composite material model. In fact it has been recently discussed that scaffolds architecture and anisotropy guide the tissue formation in the microfabricated PGS scaffolds [75]. Regarding TEHV, the FE simulations showed that material selection of the synthetic scaffold needs to be highly considered and tested in order to design and construct a TEHV that has the mechanical properties and the biocompatibility of the native tissue.

3.5 SUMMARY AND FUTURE DIRECTION

For future experiments, work is currently being continued on the geometry of the scaffold to find an ideal geometry that would produce stiffness in a cell seeded model that is close to that of native valvular tissue. The pore geometries are being changed to resemble various shapes such as a circle or square. The material properties of the scaffold are also being altered within the FE model based on other polymers such as poly lactic acid (PLA). Regarding other mechanical testing, the FE model is being used to analyze the ultimate tensile stress of the PGS scaffold and the strain to failure of the scaffold. Once the FE analysis is done, experimental testing can be done on newly microfabricated scaffolds.
4 VALVULAR INTERESTITAL CELL (VICS) SEEDED POLY (GLYCEROL SEBACATE) SCAFFOLDS: TOWARD A BIOMIMETIC BENCHMARK FOR HEART VALVE TISSUE ENGINEERING

The evaluation of candidate combinations of cells and scaffolds lacks a biomimetic in vitro model with broadly tunable, anisotropic and elastomeric structural-mechanical properties. Toward establishing such an in vitro model, in the current study, porcine aortic and pulmonary valvular interstitial cells (i.e., biomimetic cells) were cultivated on anisotropic, micro-molded PGS scaffolds (i.e., biomimetic scaffolds). Following 14 and 28 days static culture, cell-seeded scaffolds (with low and high initial cell seeding density) and unseeded controls were assessed for their mechanical properties, and cell-seeded scaffolds were further characterized by confocal fluorescence and scanning electron microscopy, and by collagen and DNA assays. PGS micro-molding yielded scaffolds with anisotropic stiffnesses resembling those of native valvular tissues in the low stress-strain ranges characteristics of physiologic valvular function. Scaffold anisotropy was largely retained upon cultivation with valvular interstitial cells; while the mechanical properties of unseeded scaffolds progressively diminished, cell-seeded scaffolds either retained or exceeded initial mechanical properties. Retention of mechanical properties in cell-seeded scaffolds paralleled the accretion of collagen, which increased significantly from 14 to 28 days. This study demonstrates that valvular interstitial cells can be cultivated on anisotropic PGS scaffolds to yield biomimetic in vitro models with which clinically relevant cells and future scaffold designs can be evaluated.

4.1 INTRODUCTION

Since the seminal work of Breuer et al. [187], Shinoka et al. [188], and Hoerstrup et al.[189], a variety of approaches have been investigated on the path toward a functional TEHV. Cells may be seeded onto s in vitro scaffolds pre-implantation [189], or captured in vivo post-implantation [190]. Scaffolds may be rendered by decellularizin gallograft valves [191, 192] or engineered tissues [193], or formed bottom-up by the cells themselves [194]. Recently, emphasis has focused on TEHV scaffold design [195-198],
bioreactor conditioning [199], minimally-invasive implantation [200], and on the vetting of potential cell sources for phenotypic mimicry and clinical relevance [139, 201-203]. Generally, native valvular tissue has served as the gold standard of structure and function. However, while comparison to the native valve is critical to assessing TEHV efficacy in vivo, earlier stages of TEHV design and testing could benefit from a more biomimetic, but altogether tissue engineered, in vitro model.

Comprising as many as five distinct phenotypes [204], valvular interstitial cells (VICs) represent a logical cell choice for a biomimetic in vitro TEHV model. Prominent in ECM formation and maintenance, VIC-mediated collagen synthesis [205] and cross-linking [206] appears to depend on transvalvular pressure, and may depend on spatial variations in cyclic VIC deformation across the circumferential-radial [207] and thickness [208] planes of the leaflet. Indeed, in vitro studies have demonstrated that VIC behavior can be modulated by substrate stiffness [209-211], mechanical load [212-214], and combinations thereof [215]. Further, clinically promising bone marrow [216] and adipose-derived [203] mesenchymal stem cells have demonstrated the potency to express VIC-similar phenotypes, including similar responses to cyclic stretch [217]. Indeed, bone marrow appears to be a source of VIC progenitors in normal valvular homeostasis [218].

However, while VICs have been investigated in various three-dimensional (3-D) scaffolds, previous 3-D constructs have been used predominately for the purpose of accommodating VICs within a more physiological culture environment. In particular, to investigate VIC phenotype [219, 220] and response to biochemical [221, 222] and physico-mechanical stimuli [127, 223]. Only recently have VIC-based 3-D constructs been structural-mechanically designed and assessed in composite, in the explicit capacity of biomimetic in vitro models and TEHVs intended for implantation [197, 198]. Combining elastomeric, tunable mechanical properties [224] with microfabricated pores amenable to structural-mechanical design, PGS [225] scaffolds rendered by laser microablation [144, 195, 226, 227] or micro-molding [92, 228] have the potential to complement VICs in a biomimetic TEHV in vitro model.
In the current study, we sought to develop a more biomimetic, tissue engineered \textit{in vitro} model for use in heart valve tissue engineering. In particular, we first developed a unique PGS micro-molding approach in which a dicing saw was utilized to generate ceramic micro-molds. As in our previous study [195], PGS scaffolds were comprised of diamond-shaped pores capable of yielding anisotropic mechanical properties. Due to the clinical need for pulmonary (PV) and aortic (AV) valve replacements, combined with valve-specific differences in VIC stiffness [205] and tissue remodeling potential [220], both porcine aortic (AVIC) and pulmonary (PVIC) valvular interstitial cells were isolated, expanded \textit{in vitro}, and dynamically seeded onto micro-molded PGS scaffolds. VIC-seeded PGS scaffolds were cultivated statically for 14 and 28 days. VIC-seeded scaffolds and unseeded controls were characterized for their mechanical properties; for comparison, porcine AV and PV leaflets were likewise tested. VIC-seeded scaffolds were further characterized by confocal fluorescence and scanning electron microscopy, and by DNA and collagen assays.

4.2 MATERIALS AND METHODS

4.2.1 PGS pre-polymer Synthesis, Micromolding Fabrication and Curing Condition

PGS pre-polymer was synthesized by polycondensation of glycerol (Sigma-Aldrich, St. Louis, MO) and sebacic acid (Sigma) under heat and vacuum, as described in detail in our previous study [195]. Of note, our synthesis methods were adapted from those introduced by Wang \textit{et al.} [224]. In brief, glycerol and sebacic acid were charged, in a 1:1 molar ratio, into a 3-neck round bottom flask. This mixture of triol and dicarboxylic acid was magnetically stirred at 120°C for 24 hours prior to an additional 24 hours stirring at both 120 °C and < 50 mTorr vacuum (to remove water vapor liberated by the polycondensation reaction, thereby driving the reaction forward). The resultant viscous pre-polymer was decanted into a container; upon cooling to room temperature, it solidified to a soft waxy consistency. PGS pre-polymer was stored at room temperature in a desiccator prior to use in solid PGS membrane or micro-molded PGS scaffold fabrication.
Toward a less expensive, higher through-put method of PGS scaffold microfabrication than our previous laser ablation methods [144, 195, 226, 227], in the present study, PGS scaffolds were fabricated by a machining-based micro-molding method, distinct from soft lithography [92] and wax printing[228]micro-molding methods previously applied to PGS. First, an approximately 0.5 inch wide by 350 µm deep edge was pre-machined around the top surface of a block of ultra-high temperature machinable glass-mica ceramic (2 x 2 x 0.5 inch; McMaster-Carr, Elmhurst, IL) using a milling machine. A precision dicing saw (Model 982-6; Kulicke and Soffa Industries, Inc., Fort Washington, PA) was then utilized in conjunction with a 90 micron-thick diamond blade (Dicemaster®; Thermocarbon, Inc., Casselberry, FL) to cut 90 micron-wide, 350 micron deep channels extending across the 1” x 1” raised surface of the ceramic block demarcated by the milled edges (Fig. 26 A). Channels were cut at angles of 30° and 120° with respect to one edge of the ceramic block, and spaced at a distance of 220 microns from the center-line of one channel to another, yielding a cross-hatched lattice of channels demarcating an array of diamond-shaped ceramic posts, each post having long-by-short axis dimensions of approximately 260 by 150 microns, respectively (i.e., ~ 1.7:1 aspect ratio, as utilized in our previous study [195]).

Dicing saw cut ceramic micro-molds were then coated with a sacrificial layer using a 90% (w/v) sucrose (Sigma) in distilled water solution and a custom-built spin-coater (3000 rpm; 30 s) [195]. Sucrose-coated molds were baked in an oven (Model 1470; VWR, West Chester, PA) at 120 °C for 18 h prior to PGS casting. In order to cast PGS pre-polymer onto sucrose-coated ceramic micro-molds, the molds were first heated to approximately 160 °C on a hotplate. Approximately 0.3 g of PGS pre-polymer was then melted and spread evenly with a stainless steel spatula around the edges of the micro-mold, directly adjacent to the central 1 x 1 inch area cross-hatched by the dicing saw. This quantity of melted PGS pre-polymer was found to readily wick into and fill the channels; the PGS was then cured in the micro-mold for 13 h at 160 °C in a vacuum oven (Model 1470; VWR) under high vacuum (< 50 mTorr; Model 1405; Welch Vacuum Technology, Niles, IL). Following curing, the assembly of cured PGS and micro-mold was soaked overnight in distilled water at room temperature to begin dissolving the sacrificial sucrose.
layer, thereby facilitating PGS detachment from the ceramic micro-mold. Delamination was achieved by positioning a razor blade against the milled edge of the mold, adjacent to the cross-hatched area and angled low (i.e., $< 45^\circ$) with respect to the surface, and then slicing through the cross-hatched area, cutting both the PGS scaffold and the embedded diamond-shaped posts from the underlying ceramic block (Fig. 26 A). The resultant diamond-shaped post fragments embedded within the PGS scaffold were removed by soaking overnight in distilled water at 60 °C, followed by gentle rinsing in distilled water.

Figure 26: (A) Schematic illustrating ceramic micro-mold fabrication by die saw cutting (A, top). The dicing saw blade and one particular cutting path are indicated by the circular disk and dashed arrow, respectively; preferred (PD) and orthogonal, cross-preferred (XD) material directions are indicated. Schematic, illustrating delamination of the PGS scaffold following 13 h curing at 160°C (A, bottom). Delamination was achieved by slicing across the surface of the mold using a razor blade and resultant diamond-shaped fragments of the ceramic mold embedded within the PGS were removed by gentle washing in distilled water. (B-C) Scanning electron micrographs (SEMs) of a representative single layer micro-molded PGS scaffold at 100x (B) and 300x (C) illustrates the fidelity of the micro-molding
technique. Scale bars = 1.0 mm and 500 microns, respectively. (D) Representative SEM of a double layer PGS scaffold assembled by laminating together two offset micro-molded PGS scaffolds. Struts comprising the bottom layer can be observed through the diamond-shaped pores of the top layer (e.g., white asterisk). Scale bar = 500 microns.

4.2.2 Assembly of Double Layered Microfabricated PGS Scaffolds

Double layered PGS scaffolds were made by adapting our previous methods [226]. Two single layers, micro-molded PGS scaffolds were first cured for 6.5 h at 160 °C and delaminated as described above. The two single layer scaffolds were then manually aligned with the assistance of an inverted light microscope, offset in such a way that the intersections of the PGS struts in the bottom layer were approximately centered beneath the centers of the pores in the top layer. The double layer PGS scaffold was then placed on a standard glass microscope slide (e.g., 75 x 25 x 1 mm; VWR), weighed down beneath an ~2 x 2 x 4 inch Teflon block (i.e., ~550 g), and cured together at 160 °C and < 50 m Torr vacuum for an additional 6.5 h (i.e., 13 h total). Both single and double layer micro-molded PGS scaffolds were autoclave sterilized for 30 min at 121 °C and 15 PSI prior to use in subsequent experiments.

4.2.3 PGS Membrane, Scaffolds and Native Tissues Mechanical Testing

Micro-molded PGS scaffolds and solid PGS membranes (cured under conditions identical to those of associated scaffolds) were mechanically tested under uniaxial tension, adapting methods we previously applied to PGS scaffolds microfabricated by laser ablation [195]. In brief, single and double layer scaffolds, as well as solid PGS membranes (~ 25 x 5 x 0.3 mm), were subjected to uniaxial tensile mechanical testing in order to measure the effective stiffness E (slope of the stress-strain curve within the 0-10% strain region; equivalent to Young’s modulus for linear elastic, homogenous, isotropic materials), the ultimate tensile strength (UTS), and the strain-to-failure (εf) using a LF Plus materials testing machine, a 5N load cell, and Nexygen™ Plus software (Lloyd Instruments, Ltd., West Sussex, UK). Of note, separate specimens were utilized for testing mechanical properties in the PD and XD directions. The
thicknesses of specimens were measured using a dial gauge (accuracy 0.01mm; The L.S. Starrett Co., Athol, MA). To prevent specimens from slipping within the grips, sandpaper was affixed to the faces of the grips prior to specimen mounting. Specimens were stretched to failure at a quasi-static rate of 7mm/min. UTS and $\varepsilon_i$ were defined as the maximum values of stress and strain reached at the onset of failure, respectively. VIC-seeded PGS scaffolds and fresh, native porcine AV and PV leaflet tissues were tested similarly, albeit wet, as opposed to dry. Separate specimens were utilized for testing native AV and PV leaflet tissues in the circumferential (CIRC) and orthogonal radial (RAD) directions; on average, test specimens were cut into 15mm long by 5 mm wide rectangular strips.

4.2.4 Cell culture, Scaffold Seeding and Construct Cultivation

4.2.4.1 Porcine VIC isolation and culture

Pig hearts (~ 9 month old) were obtained immediately following slaughter from either Brenneman's Meat Market (Huntingdon, PA) or the Penn State MeatsLaboratory (University Park, PA). Hearts were collected into an ice cold solution of 2% (v/v) antibiotic-antimycotic in Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) and transported to the lab. AV and PV leaflets were aseptically excised under a laminar flow hood and rinsed exhaustively in antibiotic-antimycotic solution to remove residual blood.

AVICs and PVICs were isolated by collagenase digestion [220]. The three leaflets from each valve were denuded of valvular endothelial cells by wiping with sterile gauze, and then digested together in 6 mL of a solution of 0.2% (w/v) type I collagenase (Worthington Biochemical, Corp., Lakewood, NJ) in HBSS at 37°C for 6-8 h in order to liberate the VICs into solution. A sterile cell strainer (100 µm mesh size; BD Biosciences, San Jose, CA) was used to remove the tissue debris, and the VICs were retrieved by centrifugation at 1000 RCF for 10 min. VICs were resuspended and expanded in a complete culture medium comprised of E199 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic (Invitrogen). VICs were utilized between passages 6 and 7.
4.2.4.2 VIC-seeding and culture of micro-molded PGS scaffolds

Autoclave sterilized micro-molded PGS scaffolds (25x 5 x 0.3 mm; n = 3 per cell type, culture duration, and orientation) were soaked in complete medium for 6 days (12 days for double layer scaffolds) to enhance cell attachment [195]. Each scaffold was placed into an individual 50 mL centrifuge tube fitted with a sterile vent filter cap (TubeSpin® Bioreactor 50; TPP Techno Plastic Products AG, Trasadingen, Switzerland). Cell suspension (12 mL) was pipetted into each tube, yielding a scaffold seeding density of ~ 5 x 10^6 VIC / cm². Scaffolds freely floated within the cell suspension and the tubes were rotated at 8 rpm for 24 h on a rotisserie (Labquake® hybridization rotator; Thermo Fisher Scientific, Waltham, MA) inside a humidified incubator operating at 37 °C and 5% CO₂ (NU-8700; Nuaire, Plymouth, MN). VIC-seeded scaffolds were subsequently transferred into individual wells of a six-well plate (Costar Ultra Low Attachment; Corning, Corning, NY) and cultivated statically for 14 or 28 days in 4 mL per scaffold of complete medium further supplemented with 82 µg/mL of L-ascorbic acid-2-phosphate sesquimagnesium salt hydrate (Sigma) to promote collagen synthesis. Control, unseeded scaffolds were likewise prepared and tested after a total of 0, 20, or 34 days incubation in medium (i.e., corresponding with the 6 days of pre-soaking plus 14 or 28 days cultivation with VICs). Control, unseeded double layer scaffolds were incubated in medium for 40 days to account for the 12 days of pre-soaking plus 28 days cultivation with VICs. Independent specimens were tested for each time point.

4.2.5 Biochemical Assays

DNA and collagen assays were conducted on VIC-seeded PGS scaffolds following 14 and 28 days cultivation, as described in our previous study [195]. In brief, DNA content was quantified using the PicoGreends DNA quantitation kit (Invitrogen) and a Spectramax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA). Collagen content was quantified using the Sircol™collagen assay kit (Biocolor Ltd., United Kingdom) and a Genesys 20 spectrophotometer (Thermo Fisher Scientific).
4.2.6 ESEM and Confocal Fluorescence Microscopy & Cell Orientation Image Analysis

Micro-molded PGS scaffolds, both bare and following 28 days VIC cultivation, were imaged as described in our previous study [195] using an FEIQuanta 200 environmental scanning electron microscope at a 20-kV accelerating voltage, working distances ranging from 10.2 to 15.2 mm, and a 0.60 Torr pressure level.

After 28 days cultivation, samples of VIC-seeded scaffold were imaged by laser scanning confocal microscopy as described in our previous study [195]. In brief, VIC-seeded scaffolds were rinsed in HBSS and fixed overnight at 2–8 °C in 10% neutral buffered formalin (Sigma), stained for filamentous (F-) actin using Alexa Fluor 488-phalloidin (Invitrogen), and cover-slipped with Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA) to counter stain cell nuclei. Samples were imaged with an x25 water-immersion objective on a Fluo-View FV1000 laser scanning confocal microscope (Olympus America, Center Valley, PA), with F-actin and nuclei pseudo-colored green and blue, respectively.

Confocal fluorescence micrographs of F-actin were analyzed utilizing a custom, Fast Fourier Transform (FFT)-based Matlab program as described previously [226]. Single, en face optical planes centered within diamond-shaped pores and located approximately 30 microns beneath the surface of the first detectable cells were analyzed (n=6 pores per cell type). The centroid of the angular orientation distribution was calculated and an orientation index (OI) was defined as the angular increment centered about the centroid encompassing 50% of the F-actin population; i.e., a lower OI indicates a tighter distribution about the centroid and thus a greater degree of alignment.

4.2.7 Statistics

Data are reported as mean ± standard error of the mean (SEM) for n ≥ 3 specimens. For PGS membranes, in which only one factor was assessed (i.e., incubation time), data were analyzed by one-way ANOVA and Tukey’s post-hoc test. For micro-molded PGS scaffolds, in which two factors were assessed
(i.e., orientation and incubation time), data were analyzed by two-way ANOVA. Likewise, for VIC-seeded scaffolds, in which two factors were assessed (i.e., cell type and culture time (for DNA and collagen) or orientation and culture time (for mechanical properties)), data were analyzed by two-way ANOVA. One AVIC and one PVIC isolation were utilized for single layer studies, while one additional AVIC and one additional PVIC isolation were utilized for double layer studies; mean ± SEM values thus represent results obtained from multiple replicates within a single study. For tangent E of the AV and PV, in which two factors were assessed (i.e., orientation and strain increment), data were analyzed by two-way ANOVA. For native AV and PV failure properties (i.e., UTS and \( \varepsilon_f \)), comparisons between CIRC and LONG orientations were made by unpaired, two-tailed t-tests. Statistics were calculated using Prism 5 software (version 5.04; GraphPad Software, Inc., La Jolla, CA).

4.3 RESULTS

Scanning electron micrographs (Fig. 26 B-D) demonstrated that the micro-molding approach (Fig. 26 A) was capable of yielding single layer PGS scaffolds with geometric features (i.e., diamond pore shape and inside dimensions, strut width) qualitatively consistent with complementary features of the associated ceramic molds. Of note, the ~ 90 µm-wide channels of the mold yielded ~ 75 µm-wide PGS struts; the difference in width was likely due to the sacrificial sucrose coating. Double layer scaffolds assembled by approximately offset alignment and bonding of two single PGS scaffolds retained similar geometric features, with the struts of the bottom scaffold layer clearly visible through the pores of the top layer scaffold (Fig. 26 D). In practice, manually achieving a uniform offset between the top and bottom layers proved difficult, due to the elasticity of the PGS struts and their tendency to bend and/or stretch.

4.3.1 Mechanical Properties of Fresh Porcine Aortic and Pulmonary Valve Leaflets

Uniaxial tensile mechanical test results for CIRC and RAD oriented specimens of native porcine AV and PV leaflet are presented in Fig. 27. Representative stress-strain curves (exponential fit, least squares regression) illustrate the nonlinear, anisotropic mechanical response of the AV (Fig. 27 A) and PV (Fig. 27 D). For the tangent E of both the AV (Fig. 27 B) and PV (Fig. 27 E), the effects of both
orientation and strain range were significant ($p < 0.0001$ and $p < 0.0001$, respectively). Of note, the tangent $E$ measured for the AV in the CIRC direction increased from a minimum of $0.05 \pm 0.02$ MPa (0.0-0.1 strain range) to a peak of $6.7 \pm 1.1$ MPa (0.4-0.5 strain range); in the RAD direction, corresponding minimum and peak tangent $E$ were $0.01 \pm 0.01$ MPa (0.0-0.1 strain range) and $1.0 \pm 0.2$ MPa (0.9-1 strain range). Similarly, the tangent $E$ measured for the PV in the CIRC direction increased from $0.07 \pm 0.03$ MPa (0-0.1 strain range) to $10.2 \pm 0.8$ MPa (0.7-0.8 strain range); in the RAD direction, corresponding values were $0.004 \pm 0.004$ MPa (0-0.1 strain range) to $0.95 \pm 0.22$ MPa (1.7-1.8 strain range). For the AV, the UTS value for RAD was significantly less than that for CIRC ($p < 0.01$), and the $\varepsilon_f$ value for RAD was significantly greater than that for CIRC ($p < 0.0001$) (Fig. 27 C). In particular, the UTS value for AV RAD ($0.50 \pm 0.09$ MPa) was 72% less than that for AV CIRC ($1.82 \pm 0.28$ MPa), and the $\varepsilon_f$ value for AV RAD ($1.13 \pm 0.05$) was 91% greater than that for AV CIRC ($0.59 \pm 0.02$). Similarly, for the PV, the UTS value for RAD was significantly less than that for CIRC ($p < 0.05$), and the $\varepsilon_f$ value for RAD was significantly greater than that for CIRC ($p < 0.01$) (Fig. 27 F). In particular, the UTS value for PV RAD ($0.74 \pm 0.18$ MPa) was 65% less than that for PV CIRC ($2.11 \pm 0.39$ MPa), and the $\varepsilon_f$ value for PV RAD ($1.60 \pm 0.12$) was 67% greater than that for PV CIRC ($0.96 \pm 0.08$).
Figure 27: Uniaxial tensile mechanical test results for circumferential (CIRC) and radial (RAD) oriented specimens of (A-C) native porcine aortic valve (AV) and (D-F) pulmonary valve (PV) leaflet. Error bars indicate standard error of the mean (SEM) for n = 5 specimens. All p-values are indicated; however, only p-values less than 0.05 were considered significant. Representative stress-strain curves (exponential fit, least squares regression) illustrate the nonlinear, anisotropic mechanical response of the AV (A) and PV (D). Tangent E were calculated for AV in strain increments of 0.1 (i.e., “0.1” refers to the strain range 0 to 0.1, etc.), up to a maximum strain range of 0.5 for CIRC and 1.0 for RAD (B). For Tangent E of the AV (B), the effects of both orientation and strain range were statistically significant (p< 0.0001 and p< 0.0001, respectively). Tangents E were calculated for PV up to a maximum strain range of 0.8 for CIRC and 1.8 for RAD (E). For Tangent E of the PV (E), the effects of both orientation and strain range were statistically significant (p< 0.0001 and p< 0.0001, respectively).Ultimate tensile strength (UTS) and strain-to-failure (εf) are presented for the AV (C) and PV (F) leaflets. For the AV (C), the UTS value for RAD was significantly less than that for CIRC (**; p< 0.01), and the εf value for RAD was significantly greater than that for CIRC (****; p< 0.0001). Similarly, for the PV (F), the UTS value for RAD was significantly less than that for CIRC (*; p< 0.05), and the εf value for RAD was significantly greater than that for CIRC (**; p< 0.01).
4.3.2 Mechanical Properties of Seeded and Unseeded Single Layer Scaffolds

Uniaxial tensile mechanical properties of single layer, micro-molded PGS scaffolds and corresponding solid PGS membranes (cured and incubated under conditions identical to those of scaffolds) are presented in Fig. 28. Specifically, scaffolds and PGS membranes were cured for 13 h at 160 °C and incubated in culture medium at 37 °C for periods of 20 and 34 days, corresponding with the 6 days of pre-soaking and culture periods of 14 and 28 days utilized in VIC-seeded scaffold experiments. For E of the scaffolds (Fig. 28 A), the effects of both orientation (p< 0.0001) and incubation time (p = 0.0003) were statistically significant. In particular, in comparison to 0 days incubation (i.e., initial mechanical properties), E of PD-oriented scaffold specimens decreased by 24.7% (i.e., from 0.55 ± 0.03 MPa to 0.41 ± 0.04 MPa) and 39.5% (to 0.33 ± 0.03 MPa) following 20 or 34 days incubation, respectively. Similarly, E of XD-oriented scaffold specimens decreased by 26.6% (i.e., from 0.17 ± 0.01 MPa to 0.13 ± 0.01 MPa) and 56% (to 0.10 ± 0.01 MPa) following 20 or 34 days incubation, respectively. A trend of decreased UTS (Fig. 28 B) was apparent for scaffold specimens; however, the effect of orientation did not quite reach statistical significance (i.e., p = 0.0586), and the effect of incubation time did not reach statistical significance. Specifically, UTS values of the PD-oriented scaffolds specimens were 0.14 ± 0.04 MPa (0 days), 0.12 ± 0.003 MPa (20 days), and 0.07 ± 0.01 MPa; corresponding UTS values for XD-oriented scaffolds were 0.08 ± 0.01 MPa (0 days), 0.07 ± 0.01 MPa (20 days), and 0.06 ± 0.02 MPa (34 days). By contrast, a trend of increased εf (Fig. 28 C) was apparent for scaffold specimens; while the effect of orientation did not reach statistical significance, the effect of incubation time was significant (p = 0.0162). In particular, in comparison to 0 days incubation, εf of PD-oriented scaffold specimens increased by 114.5% (i.e., from 0.35 ± 0.08 to 0.74 ± 0.13) following 34 days incubation; in light of the progressive increases in εf observed in XD-oriented specimens, it remains unclear if the comparatively small, 9.7% increase in εf from 0 to 20 days incubation in PD-oriented specimens was representative or resulted from a fabrication or testing-related error. In particular, εf of XD-oriented scaffold specimens increased by
20.4% (i.e., from 0.50 ± 0.06 to 0.60 ± 0.11) and 44.0% (to 0.72 ± 0.13) following 20 or 34 days incubation, respectively.

For solid PGS membranes, E decreased by 10.0% (i.e., from 1.42 ± 0.06 MPa to 1.28 ± 0.09 MPa) and 16% (i.e., to 1.19 ± 0.02 MPa) following 20 or 34 days incubation time, respectively (p < 0.05; Fig. 28 D). No clear trends were apparent for UTS (Fig. 28 E) or ε_f (Fig. 28 F) of solid PGS membrane specimens. Specifically, UTS values of solid PGS membranes were 0.65 ± 0.02 MPa (0 days), 0.53 ± 0.08 MPa (20 days), and 0.55 ± 0.06 MPa (34 days); corresponding ε_f values were 0.85 ± 0.07 (0 days), 0.75 ± 0.17 (20 days), and 0.89 ± 0.09 (34 days).

Figure 28: Uniaxial tensile mechanical test results for PD- and XD-oriented specimens of micro-molded (A-C) PGS scaffold and (D-F) solid PGS membranes incubated in culture medium at 37 °C. Error bars indicate standard error of the mean (SEM) for n = 4 specimens. All p-values are indicated; however, only p-values less than 0.05 were considered significant. For E of the scaffolds (A), the effects of both orientation and incubation time were statistically significant (p < 0.0001 and p < 0.0003, respectively). A trend of decreased UTS (B) was apparent for scaffold specimens; however, the effect of orientation did not quite reach statistical significance (i.e., p = 0.0586), and the effect of incubation time did not reach statistical significance. By contrast, a trend of increased ε_f (C) was apparent for scaffold specimens; while the effect of orientation did not reach statistical significance, the effect of incubation time was significant.
For E of solid PGS membranes (\(D\)), significant differences (\(p < 0.05\)) in comparison to 0 days incubation time are indicated by *. No clear trends were apparent for UTS (\(E\)) or \(\varepsilon_f\) (\(F\)) of solid PGS membrane specimens.

Uniaxial tensile mechanical test results for PD- and XD-oriented specimens of single layer, micro-molded PGS scaffold incubated for 6 days in culture medium (to promote cell attachment), seeded with AVIC or PVIC, and cultured for 14 or 28 days \textit{in vitro} are presented in Fig.29. For E of both AVIC- (Fig.29 A) and PVIC-seeded (Fig.29 D) scaffolds, the effect of orientation was statistically significant (\(p < 0.0001\)), while the effect of culture time was not significant, despite what appeared to be a trend of increased E with culture time in the case of the XD-oriented PVIC-seeded scaffolds (Fig.29 D). Specifically, E of the AVIC-seeded scaffolds in the PD were 0.55 ± 0.09MPa (14 days) and 0.59 ± 0.06 MPa (28 days); corresponding values for the XD were 0.12 ± 0.03 MPa (14 days) and 0.19 ± 0.01 MPa (28 days). Similarly, E of the PVIC-seeded scaffolds in the PD were 0.51 ± 0.02 MPa (14 days) and 0.54 ± 0.05 MPa (28 days); corresponding values for the XD were 0.20 ± 0.08MPa (14 days) and 0.34 ± 0.01 MPa (28 days). For UTS of both AVIC- (Fig.29 B) and PVIC-seeded (Fig.29 E) scaffolds, the effect of orientation was statistically significant (\(p = 0.0012\) and \(p = 0.0043\), respectively), while the effect of culture time was not significant. Specifically, UTS of the AVIC-seeded scaffolds in the PD were 0.16 ± 0.03MPa (14 days) and 0.18 ± 0.02MPa (28 days); corresponding values for the XD were 0.08 ± 0.003 MPa (14 days) and 0.06 ± 0.006MPa (28 days). Similarly, UTS of the PVIC-seeded scaffolds in the PD were 0.16 ± 0.02 MPa (14 days) and 0.17 ± 0.05 MPa (28 days); corresponding values for the XD were 0.09 ± 0.02MPa (14 days) and 0.09 ± 0.01 MPa (28 days). For \(\varepsilon_f\) of AVIC-seeded scaffolds (Fig.29 C), the effects of both orientation and culture time were statistically significant (\(p = 0.0067\) and \(p = 0.0129\), respectively). For \(\varepsilon_f\) of PVIC-seeded scaffolds (Fig.29 F), neither the effect of orientation nor the effect of culture time reached statistical significance. Specifically, \(\varepsilon_f\) of the AVIC-seeded scaffolds in the PD were 0.62 ± 0.16 (14 days) and 0.51 ± 0.07 (28 days); corresponding values for the XD were 0.81± 0.07 (14 days) and 0.95 ± 0.13 (28 days). Similarly, \(\varepsilon_f\) of the PVIC-seeded scaffolds in the PD were 0.46 ±
0.03MPa (14 days) and 0.72 ± 0.18 (28 days); corresponding values for the XD were 0.60 ± 0.13MPa (14 days) and 0.47 ± 0.11 MPa (28 days). Of note, the scaffolds mechanical characteristics has been compared between scaffolds seeded initially with lower and higher cell density and presented in the comparison section.

Figure 29: Uniaxial tensile mechanical test results for PD- and XD-oriented specimens of single layer, micro-molded PGS scaffold seeded and cultured with porcine (A-C) aortic valvular interstitial cells (AVIC) or (D-F) porcine pulmonary valvular interstitial cells (PVIC). For E of both AVIC- (A) and PVIC-seeded (D) scaffolds, the effect of orientation was statistically significant ($p < 0.0001$), while the effect of culture time was not significant, despite what appeared to be a trend of increased E with culture time in the case of the XD-oriented PVIC-seeded scaffolds (D). For UTS of both AVIC- (B) and PVIC-seeded (E) scaffolds, the effect of orientation was statistically significant ($p = 0.0012$ and $p = 0.0043$, respectively), while the effect of culture time was not significant. For $\varepsilon_f$ of AVIC-seeded scaffolds (C), the effects of both orientation and culture time were statistically significant ($p = 0.0067$ and $p = 0.0129$, respectively). For $\varepsilon_f$ of PVIC-seeded scaffolds (F), neither the effect of orientation nor the effect of culture time reached statistical significance. To facilitate comparison with unseeded control scaffolds incubated in culture medium, mean ± SEM values of E, UTS, and $\varepsilon_f$ for PD and XD-oriented unseeded controls are indicated by superimposed, diagonal line-marked columns. All $p$-values are indicated;
however, only $p$-values less than 0.05 were considered significant. Of note, “culture time” refers to the time following 6 days incubation in culture medium to promote cell attachment prior to seeding (i.e., “incubation time” in Fig.28 minus 6 days).

The representative stress-strain curve of cell seeded scaffolds depicted 3 categories of engineered construct (Fig.30). One group did not show that much deviation from linear elastic material however, some cell seeded scaffolds include a considerable nonlinear property, in their initial or final section of stress-strain region that is the indication of tissue formation and effect of ECM bundles formation.

Figure 30: Representative of scaffolds stress-strain curve following 28 days incubation in medium (A) showed that scaffolds retain its elastic and anisotropic characteristics and depict a linear trend of stress strain curve. While in some cases of cell seeded scaffolds, the trend of these curves completely deviated from liner manner (B-C) with linear initial stress- strain curve or non-linear initial stress-strain curves respectively. In both cases, the nonlinearity and higher UTS and ef are the indication of the presence of tissue formation and ECM. (D) In some rare cases also, the trend of stress-strain curves remained linear but with higher stiffness, UTS compared to un-seeded scaffolds that stands for the presence of tissue formation.
4.3.3 Light and Confocal Fluorescence Microscopy of Single-layer VIC Seeded Scaffolds

Following 24 hours of dynamic cell-seeding, aggregates of VICs were readily apparent within the pores, and attached to the PGS struts, of single layer, micro-molded scaffolds (not shown). Following 14, and similarly 28, days of static in vitro cultivation, single layer, micro-molded PGS scaffold pores appeared to be uniformly filled with VICs, as qualitatively observed by phase contrast microscopy (Fig.31 A). At higher magnification, confocal fluorescence micrographs of VIC-seeded scaffolds stained for F-actin and cell nuclei revealed prominent F-actin stress fibers and nuclei elongated along the directions of the stress fibers (Fig.31 B). Qualitatively, the majority of F-actin filaments appeared to extend from one strut to the nearest parallel strut. Due to the 30° and 150° orientations of the cross-hatched struts with respect to the pore long axis, F-actin filaments appeared to be preferentially aligned along the long axis of ~1.7:1 aspect ratio diamond-shaped pores, more so than along the pore short axis. While no consistent differences were readily apparent between AVIC- and PVIC-seeded scaffolds, quantification of the cell orientations by FFT-based image analysis yielded significant differences. In particular, while the centroids of the angular orientation distributions of AVIC- and PVIC-seeded scaffolds were indistinguishable and essentially aligned in parallel with the long-axis (90°) of the diamond-shaped pores (i.e., 94.7 ± 3.8° and 94.0 ± 3.4°, respectively), the OI measured for the PVIC-seeded scaffolds was significantly less than that measured for AVIC-seeded scaffolds (40.8 ± 0.9° and 52.6 ± 2.7°, respectively), suggesting that PVICs exhibited a greater degree of alignment than AVICs under the conditions tested and within the limitations of the images analyzed.
Figure 31: (A) Phase contrast (B) and confocal fluorescence micrographs of VIC-seeded scaffolds following 14 days in vitro cultivation. Following 14, and similarly 28, days of static, in vitro cultivation, micro-molded PGS scaffold pores appeared to be uniformly filled with VICs, as qualitatively observed by phase contrast microscopy (A; scale bar = 240 microns). At higher magnification, confocal fluorescence micrographs of VIC-seeded scaffolds stained for F-actin (green) and cell nuclei (blue) revealed prominent F-actin stress fibers and nuclei elongated along the directions of the stress fibers (B; scale bar = 120 microns). Note that the struts appeared blue due to PGS autofluorescence. FFT-based image analysis of confocal fluorescence micrographs (n=6 per group) was utilized to assess F-actin (i.e., cell) orientation within the diamond-shaped pores of AVIC- (C) and PVIC- (D) seeded scaffolds following 28 days culture. The centroids of the angular orientation distributions of AVIC- and PVIC-seeded scaffolds were indistinguishable and essentially aligned in parallel with the long-axis (90°) of the diamond-shaped pores (i.e., 94.7 ± 3.8° and 94.0 ± 3.4°, respectively). By contrast, the OI measured for the PVIC-seeded scaffolds was significantly less than that measured for AVIC-seeded scaffolds (40.8 ± 0.9° and 52.6 ±
2.7°, respectively), suggesting that PVICs exhibited a greater degree of alignment than AVICs under the conditions tested and within the limitations of the images analyzed.

The orientation analysis results were compared for scaffolds seeded with both cell types. While data were indicating a high oriented cells inside the diamond pores the aortic cells were slightly less oriented compared to pulmonary cells with higher OI (Fig.32 B).

![Figure 32](image)

Figure 32: (A) A circular panel were selected from inside the diamond pores (for about 16 pores from 9 images) and (B) the FFT analysis performed on all images obtained from scaffolds seeded with both aortic and pulmonary cell types and results were compared in.

### 4.1.1. DNA and Collagen Concentrations of Single-layer VIC-Seeded Scaffolds

Collagen concentration, DNA concentration, and the ratio of collagen concentration-to-DNA concentration results for specimens of single layer, micro-molded PGS scaffold incubated for 6 days in culture medium (to promote cell attachment), seeded with AVIC or PVIC, and cultured for 14 or 28 days in vitro are presented in Fig.33. For collagen concentration (Fig.33 A), the effect of culture time was significant ($p = 0.0027$); however, no significant effect was detected for cell type. In particular, in comparison to 14 days culture, collagen concentration increased 37% (i.e., from 496 ± 74 µg/g wet
weight to 681 ± 63 µg/g wet weight) and 55% (i.e., from 397 ± 38 µg/g wet weight to 615 ± 56 µg/g wet weight) for AVIC- and PVIC-seeded scaffolds, respectively. A trend of increased DNA with longer culture time was observed (Fig. 33 B); however, results did not quite reach statistical significance ($p = 0.0841$). Specifically, DNA concentrations of AVIC-seeded scaffolds following 14 and 28 days cultivation were 24 ± 4 and 41 ± 7 µg/g wet weight, respectively; corresponding values for PVIC-seeded scaffolds were 32 ± 6 and 34 ± 5 µg/g wet weight. No clear trend was apparent for the ratio of collagen concentration to DNA concentration (Fig. 33 C), with AVIC-seeded scaffolds having ratios of 21 ± 4 and 17 ± 3 µg collagen / µg DNA following 14 and 28 days cultivation, respectively; corresponding values for PVIC-seeded scaffolds were 13 ± 3 and 18 ± 3 µg collagen / µg DNA. Of note, the collagen and GAG content were considerably different between scaffolds seeded initially with lower and higher cell density (data have been presented in the comparison section).

Figure 33: (A) Collagen concentration, (B) DNA concentration and (C) the ratio of collagen concentration to DNA concentration results for specimens of micro-molded PGS scaffold incubated for 6 days in culture medium (to promote cell attachment), seeded with aortic (AVIC) or pulmonary valve (PVIC) interstitial cells, and cultivated for 14 or 28 days *in vitro*. Error bars indicate standard error of the mean (SEM) for $n = 6$ specimens. All $p$-values are indicated; however, only $p$-values less than 0.05 were considered significant. For collagen concentration (A), the effect of culture time was significant ($p = 0.0027$); however, no significant effect was detected for cell type. A trend of increased DNA with longer culture time was observed (B); however, results did not quite reach statistical significance ($p = 0.0841$). No clear trend was apparent for the ratio of collagen concentration to DNA concentration (C).
4.3.4 Data Comparison of Biochemical Assays and Mechanical Properties of Single-layered PGS Scaffolds; seeded with high and low cell density

As presented in (Fig.34) following 14 days cultivation, lower DNA and collagen was extracted from scaffolds seeded with lower cell density (e.g., collagen concentration was 496 ± 74 µg/g wet obtained from scaffolds seeded with higher AVICs density initially and was 214 ± 85 µg/g wet obtained from scaffolds seeded with lower cell density initially (1 mil./cm²) following 14 days cultivation). Similar results were found after 28 days of cultivation comparing the results between single layered scaffolds seeded with lower and higher cell density initially (5 mil./cm²) These results are also in accordance with mechanical properties of cell seeded scaffolds were scaffolds with larger collagen content were stiffer than those with lower collagen content seeded with lower initial cell density (Fig.35). These differences could be due to the fact that seeding the scaffolds with lower initial cell density results in a period which cells undergo proliferation. After cells became fully confluent on the scaffolds they undergo protein secretion and ECM generation. Whereas, cells on scaffolds seeded with high cell density, will start ECM deposition at the early stage of cultivation since the scaffolds are fully confluent with the attached cells.
Figure 34: Biochemical assays following (A-C) 2 weeks and (D-F) 4 weeks cell seeded scaffolds with both cell types. It is evident that for both cell types the collagen deposition were considerably higher with scaffolds seeded initially with higher cell density (A,D). Following 4 weeks cultivation pulmonary cell seeded scaffold reached similar amount of DNA indicating scaffolds are confluent in that stage (E). Higher values of Collagen/DNA observed for both cell types following 4 weeks scaffolds cultivation (F).
Figure 35: Mechanical characteristics of cell seeded scaffolds ((A-B) aortic and (C-D) pulmonary) compared in different group for E (A,C) and UTS (B,D). Data presented for both PD and XD orthogonal directions. The difference between low cell density and high cell density was more noticeable for aortic cell types.

4.3.5 Mechanical Properties of Seeded and Unseeded Double Layer Scaffolds

Uniaxial tensile mechanical test results for PD- and XD-oriented specimens of double layer micro-molded PGS scaffold seeded and cultured for 28 days with porcine AVIC or PVIC are presented in Fig.36. For E of both AVIC- (Fig.36 A) and PVIC-seeded (Fig.36 D) scaffolds, the effect of orientation was statistically significant ($p<0.0001$); the effect of culture time was not significant for AVIC-seeded scaffolds, but was significant for PVIC-seeded scaffolds ($p = 0.002$) (Fig.36 D). Specifically, E of the AVIC-seeded double layer scaffolds in the PD and XD were $0.83 \pm 0.08\text{MPa}$ and $0.45 \pm 0.04\text{MPa}$, respectively. Similarly, E of the PVIC-seeded double layer scaffolds in the PD and XD were $0.73 \pm 0.04\text{MPa}$ and $0.38 \pm 0.07\text{MPa}$, respectively.
0.05 MPa and 0.33 ± 0.07 MPa, respectively. For UTS of both AVIC-seeded (Fig. 36 B) and PVIC-seeded (Fig. 36 E) scaffolds, the effect of orientation was statistically significant (p < 0.0001), while the effect of culture time was not significant. Specifically, UTS of the AVIC-seeded double layer scaffolds in the PD and XD were 0.18 ± 0.02 MPa and 0.09 ± 0.01 MPa, respectively. Similarly, UTS of the PVIC-seeded double layer scaffolds in the PD and XD were 0.19 ± 0.01 MPa and 0.09 ± 0.01 MPa, respectively. For $\varepsilon_f$ of both AVIC- (Fig. 36 C) and PVIC- (Fig. 36 F) seeded scaffolds, neither the effect of orientation nor culture time were statistically significant. Specifically, $\varepsilon_f$ of the AVIC-seeded double layer scaffolds in the PD and XD were 0.37 ± 0.06 and 0.31 ± 0.03, respectively. Similarly, $\varepsilon_f$ of the PVIC-seeded double layer scaffolds in the PD and XD were 0.41 ± 0.04 and 0.43 ± 0.06, respectively.

For E of unseeded double layer scaffolds (diagonal-striped, superimposed columns in Fig. 36 A,D), both the effect of orientation and incubation time were statistically significant (p < 0.0001). Specifically, following a total of 40 days incubation to control for the 12 days soaking and 28 days culture utilized in VIC-seeded double layer scaffold experiments, E of the scaffolds decreased by 47% in the PD (i.e., from 1.1 ± 0.05 MPa to 0.58 ± 0.08 MPa) and 45% in the XD (i.e., from 0.38 ± 0.06 MPa to 0.21 ± 0.03 MPa). For UTS of unseeded double layer scaffolds (horizontal lines and confidence intervals in Fig. 36 B,E), the effect of orientation was statistically significant (p < 0.0001), while the effect of incubation time was not significant. Specifically, UTS of the unseeded double layer scaffolds in the PD and XD were 0.18 ± 0.04 MPa and 0.07 ± 0.01 MPa, respectively. For $\varepsilon_f$ of unseeded double layer scaffolds (horizontal lines and confidence intervals in Fig. 36 C,F), neither the effect of orientation nor incubation time were statistically significant. Specifically, $\varepsilon_f$ of the unseeded double layer scaffolds in the PD and XD were 0.41 ± 0.03 and 0.39 ± 0.07, respectively.
Figure 36: Uniaxial tensile mechanical test results for PD- and XD-oriented specimens of double layer micro-molded PGS scaffold seeded and cultured for 28 days with (A-C) porcine aortic valvular interstitial cells (AVIC) (D-F) or porcine pulmonary valvular interstitial cells (PVIC). Error bars indicate standard error of the mean (SEM) for n = 4 specimens. All p-values are indicated; however, only p-values less than 0.05 were considered significant. For E of both AVIC- (A) and PVIC-seeded (D) scaffolds, the effect of orientation was statistically significant (p < 0.0001); the effect of culture time was not significant for AVIC-seeded scaffolds, but was significant for PVIC-seeded scaffolds (p = 0.002) (D). For UTS of both AVIC- (B) and PVIC-seeded (E) scaffolds, the effect of orientation was statistically significant (p < 0.0001), while the effect of culture time was not significant. For εf of both AVIC- (C) and PVIC- (F) seeded scaffolds, neither the effect of orientation nor culture time were statistically significant. To facilitate comparison with unseeded control scaffolds incubated in culture medium, mean ± SEM values of E, UTS, and εf for PD and XD-oriented unseeded controls are indicated by superimposed, diagonal line-marked columns.

4.3.6 Electron Microscopy and Composition of Double-layer VIC-Seeded Scaffolds

Representative scanning electron micrographs of double layer micro-molded PGS scaffolds seeded with AVIC and cultivated for 28 days are presented in Fig.37. VICs and tissue were apparent within the diamond-shaped pores, as well as covering the visible edge of the scaffold (Fig.37 A) and spanning between pores (Fig.37 B). No significant differences were detected at 28 days cultivation
between AVIC- and PVIC-seeded scaffolds in terms of collagen concentration (599 ± 55 and 512 ± 57 µg/g wet weight, respectively; Fig.37 C), DNA concentration (34 ± 3 and 45 ± 5 µg/g wet weight, respectively; Fig.37 D), or collagen to DNA ratio (22 ± 4 and 17 ± 3 µg collagen / µg DNA, respectively; Fig.37 E).

Figure 37: (A-B) Scanning electron micrographs of double layer micro-molded PGS scaffolds seeded with AVIC and cultivated for 28 days. From the top-view (A; 250x magnification), cells and tissue were apparent within the diamond-shaped pores, as well as covering the visible edge of the scaffold (i.e., white asterisks; edge cut prior to seeding). From a rotated perspective (B; 200x magnification), cells and tissue can be seen both filling pores and in some cases spanning between pores (e.g., white arrows). Scale bars = 500 microns. (C) Corresponding collagen concentration, (D) DNA concentration, and (E) the ratio of collagen concentration to DNA concentration results. Error bars indicate standard error of the mean (SEM) for n = 20 specimens. No significant differences were detected between AVIC- and PVIC-seeded scaffolds.
4.4 DISCUSSION

Heart valves enable life sustaining unidirectional cardiac blood flow via deceptively simple check valve functions. Historically, their study has largely focused on their passive, albeit structurally-mechanically rich, ECM biomechanics, with lesser emphasis on the regulatory [229], synthetic [205], and contractile [230] roles performed by their active endothelial and interstitial cellular constituents. However, as has become progressively more appreciated through their ongoing study, heart valves are anatomically and physiologically complex across the breadth of their organ, tissue, and cellular length scales [181]. Non-viable and non-ideal, current mechanical and bioprosthetic replacement heart valves, while effective in older adults, cannot recapitulate the subtle, cell-mediated functions of healthy native valves, including growth and remodeling. As such, analogous pediatric repair and replacement strategies based on effectively non-viable cryopreserved valve homografts are typified by their impermanence, generally requiring one or more reoperations prior to adulthood to accommodate the increased outflow tract diameters commensurate with somatic growth [138].

While seemingly within our near-term technical reach based on the promising results of several large animal studies [139, 189, 201, 202], including human [191] and non-human primate [192], the development of a clinically translatable TEHV capable of years to decades-long function remains a challenging goal fraught with numerous technical obstacles and unknowns. For example, in tissue engineered pulmonary artery grafts, Kelm et al. recently reported evidence of accelerated cellular aging (i.e., decreased telomere length) by 100 days implantation in a sheep model [231]. Promisingly, however, by 240 days, telomere length in grafts was indistinguishable from that of adjacent tissue, suggesting that graft repopulation and remodeling by endogenous, host-derived cells might be sufficient for maintaining long-term in vivo function. How such results might translate to humans, however, remains to be addressed. For example, van Geemen et al. [232] recently demonstrated significant differences in the cellular and ECM compositions of TEHVs fabricated from either ovine or human cells, with more specimen-to-specimen variability in the human versus ovine constructs. Indeed, with the broad variety of
TEHV approaches currently under investigation; including different cell sources, scaffold materials and structures, and biochemical and biomechanical culture conditions; an entirely tissue engineered, biomimetic in vitro model could be useful. Particularly, because comparisons to the native valve invariably fall short, and provide little insight into the roles individual cellular and scaffold parameters contribute toward overall TEHV composition, structure and function.

Toward developing such a biomimetic in vitro model for TEHVs, in the present study, AVICs and PVICs were cultivated on anisotropic, elastomeric PGS scaffolds. While these engineered heart valve tissues remained significantly substandard with respect to native valves, their physiological relevance, controllable microstructure, and intrinsic elasticity offer the potential for systematic comparisons to clinically-relevant cell sources, alternate scaffold designs, and new biomaterials.

4.4.1 Micro-Molding of PGS Scaffolds

In general, micro-molding lends itself to high-throughput applications; entire arrays of feature scan be rendered in a single step. By contrast, laser ablation is typically implemented as a step-and-repeat process, each feature being rendered one at a time. As such, soft lithography-based micro-molding was and remains the method of choice for introducing relatively shallow, non-penetrating surface features into PGS [233]. However, when the feature in question is a through hole; a pore, for example; micro-molding is more challenging [92], both from the perspective of mold release, as well as that of mold fabrication itself. For example, in Neeley et al. [92], PGS cured on sucrose-coated poly (dimethylsiloxane) (PDMS) micro-molds was soaked in distilled water for 16 days to facilitate mold release. In the context of mold fabrication, Bian et al. devised a novel strategy by which micro-molds comprised of high aspect-ratio features (e.g., posts) could be fabricated by sequential SU-8 photo resist spin-coat / soft bake steps, yielding up to 2.5 mm tall features after 10 iterations [234]. In the case of Bian et al., however, in which PDMS replicas were used to cast cell-laden hydrogels, mold release was affected by cell-mediated contraction of the "casting" itself, with the cell-laden hydrogel essentially retracting away from the Pluronic-coated PDMS posts in such a way that the resultant engineered tissue network could be readily
removed from the mold. In a unique approach to micro-molding PGS, Kempainen and Hollister [228] generated molds using a 3-D wax printer, filled the pore space of the wax molds with a thermally curable hydroxyapatite slurry, dissolved away the wax mold, and then filled the pore space of the resultant hydroxyapatite mold with PGS. Following PGS curing, the hydroxyapatite was selectively dissolved using a decalcifying agent, leaving behind a fully 3-D PGS scaffold that was applied to cartilage tissue engineering.

Compared to our previous laser ablation methods [144, 195, 226, 227], a potential advantage of our micro-molding method was that it qualitatively appeared to yield sharper pore features (e.g., compare Fig.26 B,C to Fig.29 A,B of [195]). In our most recent study, however, finite element simulations predicted only relatively subtle differences in E between scaffolds with sharp-or round-cornered pores, with values of E in the PD predicted as 1.041 MPa and 0.908 MPa, respectively [195]. Sharp corners may, however, pose a disadvantage with respect to failure properties; our previous finite element simulations predicted peak von Mises strains within the PGS struts (under the simulated application of 0.2 macroscopic strain to the scaffold) of ~ 1.37 (PD) and ~ 0.89 (XD). These von Mises strains, predicted for scaffolds with sharp-cornered diamond shaped pores, were nearly double those predicted to manifest in pores with the slightly rounded corners observed experimentally (i.e., ~ 0.7 (PD) and ~ 0.46 (XD)). Differences in the sharpness of the corners, and previous finite element predictions of associated peak von Mises strains within the PGS struts, may help to explain differences observed between the failure properties of micro-molded and laser microfabricated scaffolds. In particular, initial UTS values for single layer micro-molded PGS scaffolds in the present study (with relatively sharp corners) were 0.14 ± 0.04 MPa (PD) and 0.08 ± 0.01MPa (XD), while corresponding values measured previously for single layer, laser microfabricated PGS scaffolds (with relatively rounded corners) were 0.4 ± 0.1 MPa (PD) and 0.14 ± 0.02 MPa (XD) (i.e., approximately double). Similar trends are apparent in the strain-to-failure data; initial εf values for single layer micro-molded PGS scaffolds in the present study were 0.35 ± 0.08 (PD) and 0.50 ± 0.06 (XD), while corresponding values measured previously for laser microfabricated PGS scaffolds were 0.62 ± 0.14 (PD) and 0.73 ± 0.12 (XD). These results and comparisons suggest that it
may be possible to predictably control the failure properties of microfabricated PGS scaffolds via subtle modifications of the pore geometries.

4.4.2 Composition and Cell Alignment of VIC-Seeded Micro-molded PGS Scaffolds

Results of DNA and collagen assays confirmed that VICs are capable of (1) modest proliferation when seeded at effectively confluent densities on micro-molded PGS scaffolds, as evidenced by the increased DNA content observed in the AVIC-seeded scaffolds at 28 versus 14 days (Fig. 33 B; 71% increase when analyzed separately from PVIC-seeded scaffolds by an unpaired, one-tailed t-test, \( p<0.05 \)), and (2) synthesizing collagen on micro-molded PGS scaffolds, as evidenced by the significantly increased collagen contents in both AVIC- and PVIC-seeded scaffolds at 28 versus 14 days (Fig. 33 A). Collagen (Fig. 37 C) and DNA (Fig. 37 D) contents of double layer scaffolds cultivated for 28 days with AVICs and PVICs were similar to those of single layer scaffolds. Further, results were collectively similar to those previously reported in TEHV-related studies involving different cells and different scaffolds. For example, Engelmayr et al. previously cultivated ovine vascular smooth muscle cells on 50:50 blend nonwoven poly (glycolic acid):poly (L-lactic acid) scaffolds for a period of 3 weeks (i.e., 21 days) under static and bioreactor-mediated cyclic flexure conditions [51]. By 21 days, collagen contents were 546 ± 111 μg/g wet weight for the statically incubated samples and 893 ± 133 μg/g wet weight for the cyclically flexed samples. These values bracket the 28 day collagen concentration results obtained for AVIC- and PVIC-seeded single layer PGS scaffolds in the current study (i.e., 681.1 ± 63 and 615.1 ± 56 μg/g wet weight, respectively). In a more recent study, Engelmayr et al. reported collagen concentrations for nonwoven scaffolds cultivated for 3 weeks (i.e., 21 days) with ovine bone marrow-derived mesenchymal stem cells, with values of 422 ± 98 μg/g wet weight (static culture), 530 ± 106 μg/g wet weight (cyclic flexure), 498 ± 95 μg/g wet weight (laminar flow), and 844 ± 278 μg/g wet weight (combined cyclic flexure and laminar flow) [235]. In the context of microfabricated PGS scaffolds similar to those used in the current study, we recently measured collagen and DNA contents of 736 ± 193 μg/g wet weight and 17 ± 4 for 1.7:1 μg/g wet weight, respectively for laser microfabricated PGS scaffolds comprised of 1.7:1
aspect ratio diamond-shaped pores and cultivated for 21 days with neonatal rat dermal fibroblasts[195]. Collectively, however, these collagen concentrations measured for engineered heart valve tissues were far lower than those of the native porcine AV and PV. In particular, Huang et al. recently measured collagen concentrations of the porcine AV and PV using the same collagen assay kit used in the current study; values ranged from ~ 60,000 μg/g wet weight in the AV belly region to ~ 90,000 μg/g wet weight in the PV free edge region [236].

FFT-based image analysis of confocal fluorescence micrographs of F-actin demonstrated quantitatively that VICs preferentially align along the long-axis of the diamond-shaped pores. Moreover, PVICs demonstrated a significantly higher degree of alignment under the conditions tested and limitations of the images analyzed. Engelmayr et al. previously demonstrated that increasing the aspect ratio of microfabricated non-degradable scaffolds comprised of rectangular pores could significantly enhance the orientation of cells (rat skin fibroblasts) and their synthesized collagen along the long axis direction of the pores [237]. OI values were calculated from small angle light scattering (SALS) data and yielded values ranging from 25° to 65° (i.e., bracketing the values measured in the current study). Further, Engelmayr et al. demonstrated in that study that 2:1 aspect ratio diamond shaped pores could qualitatively promote cell orientation along the pore long axis [32]. More recently, Engelmayr et al. investigated cell alignment within PGS scaffolds with elongated, accordion-like honeycomb pores, 2:1 aspect ratio rectangular pores, and square pores. Scaffolds were fabricated by laser microablation and seeded with a mixed population of heart cells, including cardiomyocytes and cardiac fibroblasts. OI values measured using the Matlab program utilized herein ranged among 85 ± 5° for square pores, 54 ± 1° for accordion-like honeycomb pores, and roughly 40° for adult rat right ventricular myocardium (a control tissue)[226]. Guillemette et al. recently investigated the orientation of C2C12 skeletal muscle cells on laser microfabricated PGS scaffolds comprised of rectangular or square pores with or without a supplementary contact guidance grooves [144]. C2C12 cells were found to preferentially align along the long axis of the rectangular pores (as measured from scanning electron micrographs), with a significantly greater degree of alignment.
observed in scaffolds exhibiting the contact guidance grooves. In particular, approximately 60% of the C2C12 cells were aligned within ± 10° of the rectangular pore long axis with the supplementary contact guidance grooves. By contrast, less than 20% of the C2C12 cells were aligned in the case of the square pore without the contact guidance grooves. Results of Guillemette et al. [144] suggest that the addition of contact guidance features to the diamond shaped pores could potentially enhance preferential alignment of the seeded VICs.

4.1.2. Mechanical Properties VIC-seeded of Micro-molded PGS Scaffolds

A comparison of E and UTS values between unseeded (Fig.28 A) and VIC-seeded (Fig.29 A,D) scaffolds clearly demonstrates that (1) unseeded micro-molded PGS scaffolds incur degradation of E and UTS values over time and (2) single layer VIC-seeded scaffolds either retain or exceed their initial values of E and UTS, up to at least 28 days incubation. Of note, the "culture times" in Fig.29 represent the times during which VICs were present on the scaffolds; the single layer VIC-seeded scaffolds were pre-incubated in complete culture medium for 6 days prior to seeding, yielding the 20 (i.e., 6 + 14) and 34 (i.e., 6 + 28) day "incubation times" utilized for control, unseeded scaffolds in Fig.29. Given previous demonstrations of the positive reinforcing effect cell-secreted collagen can have on E of a scaffold [51], it is not surprising that the ~ 25 % (20 days) and ~ 50 % (34 days) decreases in E observed following incubation of the unseeded single layer scaffolds appeared to be entirely offset in the single layer VIC-seeded scaffolds in parallel with the progressive accretion of collagen on the scaffolds. By contrast, despite accumulating collagen concentrations similar to those of VIC-seeded single layer scaffolds, both AVIC- and PVIC-seeded double layer scaffolds demonstrated decreases in E and UTS in the PD-oriented specimens following 28 days culture (Fig.39 A,D; E decreases of ~25 % and ~34 %, respectively). This was in contrast to XD-oriented double layer scaffold specimens, in which no comparable decreases were observed. We tentatively speculate that the decreases in E observed in the PD specimens may have been due to partial de-bonding of the two scaffold layers. In particular, the nearly two-fold higher initial E exhibited by the PD-oriented double layer (1.1 ± 0.05 MPa) versus single layer (0.55 ± 0.03 MPa)
scaffold is expected, due to the offset alignment and bonding of the two layers and associated rigidifying effect of the effectively denser strut structure. Indeed, following 28 days culture, E values of the VIC-seeded double layer scaffolds in the PD direction remained higher than those of the VIC-seeded single layer scaffolds. These results suggest that the structural-mechanical effects of offset bonding and the strength of adhesion between PGS scaffold layers, warrant further investigation.

In order to estimate the increase in E that the collagenous tissue forming within the pores contributed to the degrading single layer scaffold, a simple rule-of-mixtures model, modified to account for the angular orientation of the PGS struts with respect to the loading axes, can be utilized [238]:

\[ E = E_{PGS}v_{PGS} \cos^2 \theta + E_{ECM}v_{ECM} \]

where E refers to the measured effective stiffness of the composite VIC-seeded scaffold, \( E_{PGS} \) is the stiffness of the PGS comprising the scaffold struts, \( v_{PGS} \) is the associated PGS volume fraction, \( \theta \) is the angle the PGS struts make with the loading axis, \( E_{ECM} \) is the stiffness of the collagenous tissue forming within the pores, and \( v_{ECM} \) is the associated ECM volume fraction. Based on the approximate dimensions of the pores and PGS struts, it can be readily shown that \( v_{PGS} \approx 0.6 \). Using the initial mean value of \( E_{PGS} \) measured for the PGS membrane (i.e., 1.42 MPa), and the “cosine-squared” correction factors to account for \( \theta = 30^\circ \) (PD) and \( \theta = 60^\circ \) (XD) (i.e., factors of 0.75 and 0.5, respectively), the rule-of-mixtures estimates for E of the unseeded single layer micro-molded PGS scaffold in the PD and XD can be obtained by the product of these three values, yielding 0.64 MPa (PD) and 0.43 MPa (XD). The PD estimate is clearly similar to the experimentally measured value of 0.54 ± 0.03 MPa, while for the XD, the rule-of-mixtures appears to significantly overestimate the experimentally measured value of 0.17 ± 0.01 MPa. Nevertheless, assuming the same value (i.e., 0.6) for \( v_{PGS} \), but using the mean values of \( E_{PGS} \) measured for the PGS membranes after 20 (i.e., 1.28 MPa) and 34 days (1.19 MPa) incubation, the rule-of-mixtures estimates for E of the degrading scaffold in the PD are 0.58 MPa (20 days) and 0.54 MPa (34 days). In contrast to the reasonably good estimate of the initial value of E measured in the PD, these values appear to significantly overestimate the experimentally measured values of 0.41 ± 0.04 MPa (20 days).
and 0.33 ± 0.03 MPa (34 days). A likely contributor to these differences is the higher surface area to volume ratio presented by the porous PGS scaffold compared to the solid PGS membrane. Based on the dimensions of the PGS membrane test specimens (i.e., ~ 25 x 5 x 0.3 mm), and on the dimensions of the pores and PGS struts comprising the scaffolds, the surface area to volume ratios can be readily calculated as 0.007 µm\(^{-1}\) (solid PGS membrane) and 0.027 µm\(^{-1}\) (porous PGS scaffold); i.e., the surface area to volume ratio of the single layer micro-molded PGS scaffold is ~ 3.8 times as large as that of the solid PGS membrane. Given that PGS has been reported to degrade principally via surface hydrolysis [239], the higher surface area to volume ratio presented by the scaffold likely resulted in a greater degree of PGS degradation per unit volume than in the solid PGS membrane. Indeed, if the 10% and 16% decreases in the value of E measured for PGS membranes following 20 and 34 days incubation are multiplied by 3.8, to estimate \(E_{PGS}\) for the PGS comprising the struts of the micro-molded scaffold (i.e., 0.9 MPa (20 days) and 0.54 MPa (34 days)), the rule-of-mixtures estimates for E of the single layer micro-molded scaffold in the PD become 0.41 MPa (20 days) and 0.24 (34 days), very similar to the experimentally measured values.

These rule-of-mixtures models can now be utilized to estimate \(E_{ECM}\); the effective stiffness of the collagenous ECM within the pores of the VIC-seeded PGS scaffolds. As a first approximation, scaffold degradation in the VIC-seeded scaffold can be assumed to be approximately equal to that measured in the control, unseeded scaffolds incubated in culture medium. Using the mean values of E measured for the unseeded and AVIC-seeded scaffolds, and \(v_{ECM} \approx 0.4\) (i.e., 1 - 0.6), \(E_{ECM}\) is estimated as 0.26 MPa (14 days) and 0.52 MPa (28 days). These rule-of-mixtures estimates for \(E_{ECM}\), corresponding with collagen concentrations of 496 ± 74 µg/g wet weight (14 days) and 681 ± 63 µg/g wet weight (28 days), are higher than 0.13 MPa, which was the maximum value of E estimated for the ECM within vascular smooth muscle cell-seeded nonwoven scaffolds cultivated under bioreactor-mediated cyclic flexure for 3 weeks (collagen concentration of 893 ± 133 µg/g wet weight) [238]. Thus, the specific stiffnesses (i.e., stiffness per concentration) of the collagenous ECM within the diamond-shaped pores of the AVIC-seeded micro-
molded PGS scaffolds, ~ 0.50 kPa / µg/g wet weight (14 days) and ~ 0.76 kPa / µg/g wet weight, appear to be five to seven times higher than the specific stiffness of the collagen estimated in 3 week cyclic flexure case cited above (i.e., 0.09kPa / µg/g wet weight). Given the significant differences in microstructure between the micro-molded and nonwoven scaffolds, the apparent collagen specific stiffnesses likely depend both on intrinsic properties of the collagen itself (e.g., type, fiber diameter, cross-linking), as well as scaffold-specific interactions between the collagen and the scaffold microstructural elements.

4.4.3 Native Porcine Aortic and Pulmonary Valve Leaflet Properties

Mechanical properties measured in the current study for native porcine AV leaflets were consistent with previously reported values in the literature. For example, Mavrilas and Missirlis reported peak tangent E values of 7.78 ± 1.7 MPa and 1.28 ± 0.34 MPa for the CIRC and RAD directions of fresh porcine AV leaflets, respectively [240]. Corresponding values measured in the current study were 14 % lower (6.7 ± 1.1MPa) and 22 % lower (1.0 ± 0.21MPa). By contrast, Mavrilas and Missirlis reported that human AV leaflets were stiffer, particularly in the CIRC direction, with peak tangent E of 14.55 ± 3.7 MPa (CIRC) and 1.57 ± 0.1 MPa (RAD) [240]. Comparatively little information is available in the literature regarding mechanical properties of the native porcine PV leaflets [241, 242]. In the present study, the peak tangent E of the PV leaflet tissue was found to be higher than that of the AV in the CIRC direction and similarly stiff compared to the AV in the RAD direction. Specifically, compared to the AV, the PV peak tangent E was measured to be 52 % higher in the CIRC direction (10.2 ± 0.80MPa) and 5 % lower in the RAD (0.95 ± 0.22 MPa). In both the CIRC and RAD directions, the UTS of the PV was measured to be higher than that of the AV. Specifically, in the CIRC direction the UTS of the PV (2.11 ± 0.39MPa) was measured to be 16 % higher than that of the AV (1.82 ± 0.28MPa); in the RAD direction, the UTS of the PV (0.74 ± 0.18 MPa) was measured to be 46 % higher than that of the AV (0.50 ± 0.09 MPa). Similarly, in both the CIRC and RAD directions, $\varepsilon_f$ values were measured to be higher for the PV than the AV. Specifically, in the CIRC direction the $\varepsilon_f$ of the PV (0.96 ± 0.08) was measured to be 62 %
higher than that of the AV (0.59 ± 0.02); in the RAD direction, the ε_r of the PV (1.60 ± 0.12) was measured to be 42% higher than that of the AV (1.13 ± 0.05). Results for the PV differed from a previous study by Stradins et al. comparing the human semilunar valve leaflets [243]. Specifically, while we observed a higher peak tangent E in the PV versus AV leaflet CIRC and RAD specimens, Stradins et al. did not detect any significant CIRC differences in the respective human valve leaflets, reporting 16.05 ± 2.02 MPa (PV CIRC) and 15.34 ± 3.84 MPa (AV CIRC), with similarly comparable UTS and ε_r values. By contrast, Stradins et al. found modest differences in the RAD oriented specimens, with a significantly lower peak tangent E in the PV (1.32 ± 0.93 MPa) versus AV (1.98 ± 0.15 MPa) leaflet specimens (p<0.02) [243]. In light of the nearly two-fold higher peak tangent E exhibited by human versus porcine semilunar heart valve leaflets in the CIRC direction, differences between the PV and AV leaflets are likely species specific and will likely need to be accounted for in translating results of animal studies to humans.

Compared to either the native AV or PV, the mechanical properties of the VIC-seeded micro-molded PGS scaffolds following 28 days culture were significantly substandard in terms of peak tangent E, UTS, and ε_r. However, the strain magnitudes at which native leaflets exhibit their peak tangent E, UTS, and ε_r under uniaxial tensile testing are far above the physiological strain ranges the valve leaflets experience during normal functioning. For example, the E value of the single layer AVIC-seeded scaffold following 28 days culture (0.59 ± 0.06 MPa) was found to be comparable to the tangent E of the native AV in the CIRC direction within the 10-20% strain range (0.49 ± 0.18 MPa). Based on previous observations of significant increases in E in human cell-based TEHV fabricated from more rapidly degrading nonwoven poly(glycolic acid)[244], it is anticipated that VIC-seeded micro-molded PGS scaffolds would undergo further remodeling of mechanical properties with extended culture as the scaffold progressively degrades away and the collagenous ECM accumulates.
4.4.4 Limitations and Future Directions

The tensile mechanical response of native heart valve leaflets is not only anisotropic, but also highly non-linear (e.g., Fig.27); as such, a limitation of our current micro-molded PGS scaffolds, and previous laser microfabricated PGS scaffolds [195], remains the essentially linear mechanical response of the scaffold itself. In future studies, the PGS microstructural elements comprising the scaffold (i.e., the struts) could be rendered thinner, either directly through adjustment of microfabrication parameters, or post-fabrication, by lipase-mediated surface hydrolysis [239]. At strut width to inside strut length ratios below 0.25, a transition from strut bending to strut axial extension would be expected to manifest upon progressive scaffold stretch [245], with associated increases in tangent E (i.e., non-linearity). Of note, while in the present study 90 µm-wide dicing saw blades were utilized to cut the cross-hatched channels of the micro-mold, blades as thin as 50 µm are commercially available (Thermo carbon); given that 90 µm-wide blades yielded ~75 µm-wide struts (likely due to the sacrificial sucrose coating), 50 µm-wide struts would be expected to yield ~35 µm-wide struts, which, based on the current strut inside length of 150 µm, would be expected to yield a strut width to inside strut length ratio of ~0.23 (i.e., < 0.25; offering the possibility of a non-linear tensile mechanical response). Further, given the significant increases in collagen content observed from 14 to 28 days cultivation (Fig.33A), it is anticipated that upon extended culture and associated collagen accumulation and PGS degradation, that the mechanical response of the construct would shift from the scaffold to that of the non-linear, de novo synthesized collagenous ECM, as has been observed in TEHVs based on human cells and more rapidly degrading poly(glycolic acid) scaffolds [244]. In the present study we did not directly quantify PGS degradation; rather, we inferred degradation from changes in mechanical properties. Of note, while decreases in E and UTS typify the degradation behavior of most polymers, the increases in $\varepsilon_f$ observed previously [226] and in the present study can be attributed to the 3-D network structure of PGS, which is more extensible at lower cross-link densities regardless of whether the lower cross-link density was arrived at by lower temperature synthesis, shorter curing time, or as a result of degradation. Previous studies have explicitly
focused on the degradation behavior of PGS *in vivo* [239, 246] and *in vitro* [239, 247]. While PGS has been shown to degrade primarily by enzyme-mediated surface hydrolysis *in vivo*, with normalized mass loss rates of approximately 20 mg/cm²-week in a subcutaneous implantation model [239], *in vitro* degradation includes contributions from surface hydrolysis (particularly in the presence of lipase enzymes [239]) and bulk hydrolysis associated with polymer network swelling [247]. In the present study, we did not observe evidence of gross changes in PGS strut dimensions following incubation with or without VICs; however, precise monitoring of PGS strut dimensions during incubation clearly warrants investigation in future studies.

Another limitation of our current and previous [195] studies was the use of uniaxial versus biaxial testing. While uniaxial testing is sufficient to discern directional differences in the mechanical properties of anisotropic, planar soft tissues, biaxial testing is generally considered essential to formulating constitutive equations capable of fully describing the mechanical response of such tissues under generalized loading conditions [248]. With the recent commercialization of turnkey biaxial test systems and their application to heart valve leaflets [236], it is anticipated that such equipment will become more readily available to tissue engineers in the future.

A limitation of our current dicing saw-based micro-molding method is that the ceramic micro-molds are not reusable; the diamond-shaped ceramic posts that comprise the mold proper are sliced off the underlying block of ceramic along with the PGS scaffold (Fig. 26 A). Extended soaking in water, such as the 16 days previously utilized by Neeley et al. [92], could potentially allow for PGS scaffolds to release from the micro-molds without tearing, thereby allowing for re-use of the molds; however, based on the degradation in mechanical properties observed when micro-molded PGS scaffolds were incubated for 20 and 34 days in culture medium at 37 °C (Fig. 28 A), it may be preferable to retain initial mechanical properties by using disposable molds.

Finally, another limitation of our current *in vitro* model is that the gene expression profiles of the seeded AVICs and PVICs were not characterized following isolation or serial passaging. As demonstrated by Liu et al., VICs represent a mixed population comprising as many as five distinct phenotypes [204]; it
is possible that the relative proportions of these various phenotypes may change during serial passaging, and that such potential changes could influence AVIC- and PVIC-mediated tissue formation. As such, the mechanical property, DNA, and collagen values reported in the present study should be interpreted within the specific context of the passage 6-7 porcine AVICs and PVICs utilized herein. Further, by virtue of the critical role of the valvular endothelium in maintaining a non-thrombogenic blood-tissue interface, as well as the regulatory role of valvular endothelial cells demonstrated by Butcher and Nerem in an in vitro 3-D co-culture with VICs [249], endothelialization warrants investigation in future experiments.

4.5 CONCLUSION

Toward establishing a biomimetic, tissue engineered in vitro model for the purpose of complementing native valvular tissues in the rational assessment of TEHV properties, in the current study, porcine AVICs and PVICs were successfully cultivated for 14 and 28 days on anisotropic, micro-molded PGS scaffolds, demonstrating cellular alignment along the long axis of diamond-shaped pores and retention of anisotropy and stiffness in association with the accumulation of VIC-secreted collagen. Future studies utilizing these VIC-seeded PGS scaffolds will be designed to capitalize on the intrinsic elasticity conferred by their PGS material-of-construction, with aims of further characterizing the roles of physiologically relevant mechanical stimuli (e.g., flexure, stretch, and flow [235]) in promoting engineered heart valve tissue formation by VICs.
5 ELECTROSPUN PGS: PCL MICROFIBERS ALIGN HUMAN VALVULAR INTERSTITIAL CELLS AND PROVIDE TUNABLE SCAFFOLD ANISOTROPY

The development of biomimetic scaffolds is a major challenge in heart valve tissue engineering. One of the most important structural characteristics of mature heart valve leaflets is their intrinsic anisotropy, which is derived from the microstructure of aligned collagen fibers in the extracellular matrix. In the present study, we used a directional electrospinning technique to fabricate fibrous PGS:PCL scaffolds containing aligned fibers, which resemble native heart valve leaflet ECM networks. In addition, the anisotropic mechanical characteristics of fabricated scaffolds were tuned by changing the ratio of PGS:PCL to mimic the native heart valve’s mechanical properties. Primary human valvular interstitial cells (VICs) attached and aligned along the anisotropic axes of all PGS:PCL scaffolds with various mechanical properties. The cells were also biochemically active in producing heart valve-associated collagen, vimentin, and smooth muscle actin as determined by gene expression. The fibrous PGS: PCL scaffolds seeded with human VICs mimicked the structure and mechanical properties of native valve leaflet tissues and would potentially be suitable for the replacement of heart valves in diverse patient populations.

5.1 INTRODUCTION

To engineer functional heart valve leaflets, the constructed scaffolds should: (a) mimic the anisotropic mechanical properties of native heart valve leaflets [9, 46], (b) have a fibrous structure containing aligned fibers, resembling the microstructure of the native tissue [250, 251], (c) have elastic characteristics and similar to native tissue deformation [11, 252], and (d) possess controlled degradation and support tissue regeneration at a rate to maintain structural integrity. [37, 47]. One tissue engineering approach to creating heart valves uses scaffolds as temporary supports. However, previously fabricated scaffolds for tissue TEHV have had some limitations, including plastic deformation [57], high stiffness(e.g. non-woven scaffolds) [50, 55, 122], non-anisotropic properties (e.g. homogenous structural-mechanical properties in fibrin gels) [41, 58], and low stability or lack of suturability (e.g. hydrogel based
The functionality of heart valve tissue is derived from its complex architecture. Recently, significant attempts have been made to recapitulate this complexity \textit{in vitro} while providing a suitable environment for cell proliferation and ECM production [40, 45-47]. Several of these studies have fabricated scaffolds for TEHV by using synthetic materials. For example, Gottlieb \textit{et al.} used non-woven scaffolds composed of PGA and PLLA as TEHV. Despite promising results \textit{in vitro}, these scaffolds stiffened \textit{in vivo} over time [44, 52]. In another study, Masoumi \textit{et al.} generated microfabricated scaffolds from highly elastic PGS for TEHV. The fabricated elastomers had an anisotropy and elastic modulus similar to native pulmonary heart valves (~4MPa). However, the scaffolds were limited by their non-fibrous structures and the absence of interconnected micropores within 3D constructs to support tissue formation [45, 46].

Our group has recently fabricated elastic fibrous scaffolds containing random fibers by electrospinning PGS and PCL to produce TEHV constructs [47, 57] with an elastic modulus in the range of 8.5 MPa that mimicked the mechanical stiffness of the native heart valve [57]. In addition, these composite materials supported the attachment and growth of porcine valvular interstitial cells (VICs). The PGS:PCL scaffold were associated with increased production of collagen compared to pure PCL [47]. However, these scaffolds were unable to simulate the anisotropic characteristics of the native heart valve.

In addition to the fabrication of suitable TEHV scaffolds, the use of appropriate cell types may play an important role in heart valve regeneration. VICs have been used for engineering heart valves due to their presumed ability to synthesize appropriate ECM [46, 47]. However, in previous studies, nonhuman VICs have been used, which other studies have shown can differ from human VICs in phenotypic and biochemical responses [57, 254]. Therefore, it is important to study the interaction of human VICs and biomaterials when designing scaffolds for clinical applications.

In this study, we aimed to fabricate elastic PGS:PCL scaffolds using directional electrospinning to induce human aortic VICs proliferation and maturation. We hypothesized that PGS:PCL scaffolds...
containing aligned fibers could provide anisotropic mechanical properties similar to native heart valves. The scaffolds of polymer composites were expected to provide biocompatibility, durability, and biodegradability. The tunable mechanical properties of fabricated PGS:PCL scaffolds provide the ability to mimic heart valve leaflets with various mechanical properties. The capability of fabricated composites to support viability, attachment, proliferation, and protein expression of aortic human and porcine VICs was assessed.

5.2 MATERIALS AND METHODS

5.2.1 Fabrication of PGS:PCL Scaffolds Containing Aligned and Random Fibers

PGS pre-polymer was synthesized based on previously reported process (M\text{w}=10,000) [45, 145]. Briefly, sebacic acid and glycerol with 1:1 molar ratio were reacted at 120°C under high vacuum (~50mTorr) for 24 hr to synthesize PGS pre-polymer. Various ratios of PGS and PCL blends (e.g. 1:4, 1:2, 1:1, and 2:1) were then dissolved in an anhydrous chloroform:ethanol (9:1) solution with the overall polymer concentration of 20\%(w/v). To obtain a homogenous mixture, the polymer solutions were allowed to mix overnight at room temperature prior to the electrospinning process.

A typical electrospinning system was employed to spin the pre-polymers into sheets (Fig.38 A). The pre-polymer mixture was pushed via syringe pump at a flow rate of 2 mL/hr and electrospun at 18 kV. The distance from the tip of the gauge needle to the collector plates was set at 18 cm [57]. To form electrospun sheets with randomly distributed fibers, the fibers were spun onto a glass slide placed on a grounded metallic base. Aligned fibers were created by electrospinning the fibers between two grounded electrodes, separated by 1.5 cm. The microfibers were then desiccated in a vacuum chamber overnight before further characterizations. Importantly, hydrophilicity was improved in scaffolds by immersion in 0.05M NaOH for 5 min followed by copious washing step with PBS.
Figure 38: (A) Schematic diagram of scaffold fabrication process and polymer composition. Two electrospinning techniques were used to design the scaffolds with random and aligned fibers. The random fibers were collected on a cylindrical metal plate and aligned fibers were collected between two aluminum electrodes. The randomly deposited fibers resulted in a random cellular orientation and aligned fibers resulted in cell alignment. (B) Molecular structure of PGS and PCL representing the ester bonds and alkaline groups. (C) FTIR spectra of the fibrous scaffolds with different ratios of PGS:PCL (2:1, 1:1, 1:2, and 1:4) compared with pure PCL and pure PGS sheets.

5.2.2 Physical Characterization of Microfibrous Scaffolds

The chemical compositions of the PGS:PCL scaffolds were evaluated by performing Fourier-transform infrared spectroscopy (FTIR) analysis (Bomem, MB 100) over a range of 500 - 4000 cm\(^{-1}\) and resolution of 2 cm\(^{-1}\). In addition, the wettability of the composite scaffolds was tested by using contact angle measurement with static drop technique (video contact angle system, VCA Optima, AST Inc.) [57]. Prior to obtaining scanning electron microscopy (SEM) images the samples were sputter-coated by a thin layer of gold and palladium. The fiber diameters and pore sizes for both aligned and random scaffolds
were measured by importing the related SEM images into Image J software (NIH).

*In vitro* degradation rate was assessed based on the weight loss of the scaffolds, measured for 10 days in 5 ml Dulbecco’s Phosphate Buffered Saline (DPBS) (GIBCO) with 0.5mM sodium hydroxide (NaOH) at 37 °C. Four PGS:PCL samples with a 5 mm diameter and thickness of 150 µm were cut with a biopsy punch and were used to determine the degradation rate. The scaffolds were then rinsed in DPBS and dried following each time points. The samples’ weights were then measured and the percentage of mass loss was calculated by dividing the weight loss to the initial dry weight for each sample.

The tensile properties of samples with aligned and random fibers were tested under uniaxial tension using a mechanical tester (Instron, Model 5542, Norwood, MA) with 10 N load capacity and at a constant rate of 7 mm/min. The data were processed using Blue Hill software (Instron), which measured the force/deformation and stress-strain curves for each sample. Rectangular samples with a length of 10 mm, width of 5 mm, and a thickness of 0.1 mm were stretched in both dry and wet conditions for comparison. We calculated the effective stiffness of the scaffolds from the initial 0-15% strain region of stress/stain curves (the slope E, is corresponding to Young’s modulus defined for elastic homogenous materials). The ultimate tensile strength (UTS), the maximum stress, and strain to failure (εf) were also obtained from the stress–strain curves. The mean and standard deviation values of 4-6 independent measurements were reported and considered for comparison. For structures with aligned fibers, the samples were tested into two directions; one aligned with the fiber’s direction, referred to as the preferred direction (PD) and the other one perpendicular to the fiber alignment, referred to as the cross-preferred direction (XD). The anisotropic mechanical characteristics of the aligned fibers for each ratio were also calculated by dividing the average value in the PD direction by the XD direction.
5.2.3 **In Vitro Studies**

5.2.3.1 **VIC Isolation and Characterization**

In this study, we cultured both human and porcine VICs on our microfibrous electrospun sheets. All cell culture supplies were purchased from Invitrogen (CA, USA). Human aortic VICs were obtained from the Department of Cardiac Surgery at Boston’s Children Hospital. Aortic VICs were isolated from fresh aortic valve leaflets excised under sterile conditions from transplant recipients’ explanted hearts with normal valve leaflets. Leaflets were washed several times in cold PBS before the endothelium was scraped off and the remaining tissue was minced with a blade to facilitate matrix digestion. It was then incubated at 37°C for 1 hr in a digestion buffer containing 10X calcium/magnesium buffer, 0.2% (v/v) type I collagenase (Roche), and 2.5 U/ml dispase (BD Bioscience) in culture medium containing Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1% (v/v) antibiotic-antimycotic (Invitrogen). The digested tissue solution was then centrifuged at 1200 rpm for 5 min, resuspended in culture medium and plated on tissue culture plastic. Isolated VICs were expanded and utilized between passage 3 and passage 7.

Porcine VICs were also isolated by collagenase digestion[26] from AV porcine leaflets, which were aseptically removed from donated hearts (Children’s Hospital, Boston, MA). The valvular endothelial cells were first removed by wiping the leaflet surface. The tissue was then digested by using 0.5% (w/v) type I collagenase in Hank's Balanced Salt Solution (HBSS) at 37°C for 6-8 h. The solution containing cells was centrifuged at 1200 rpm for 10 min and the isolated porcine VICs were expanded and utilized between passages 6 and 7.

5.2.3.2 **Cell Seeding**

PGS:PCL scaffolds with 5 mm diameter and 100 μm thickness were used for cell seeding. The samples were soaked in 70% ethanol for 30 min followed by high intensity UV exposure (800 mW) for 2 min. Prior to cell seeding, the scaffolds were soaked in DMEM media overnight. Each scaffold was
seeded with 50,000 cells suspended in 20μl of DMEM. The plates were incubated for 3hr initially to allow cells to attach the scaffold surface and then 200 μl media were added to each well. The media was then changed every other day.

5.2.3.3 Cell Attachment

The cell attachment to the scaffolds was quantified by counting the number of the cells adhered to the scaffolds at 12 hr post-seeding. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Invitrogen) for visualization. Twelve hours after the initial cell seeding, three scaffolds from each ratio of PGS:PCL were washed twice with DPBS, fixed in 4% paraformaldehyde (PF) solution in DPBS for 20 min at room temperature, and stained with DAPI. Fluorescent images were then taken from 10 regions of each scaffold surface using an inverted microscope (Nikon TE 2000-U, Nikon instruments Inc., USA). The images were analyzed by Image J software to count the number of cells per unit area.

5.2.3.4 Viability Assay

Cell viability was determined at days 1 and 10 using Live/Dead assay kit (calcein-AM/ethidium Bromide homodimer; Invitrogen) according to the manufacturer’s protocol. Three scaffolds from each composition were washed with PBS and incubated in live/dead solution for 20 minutes. Fluorescent images were then taken by using an inverted fluorescence microscope (Nikon TE 2000-U, Nikon instruments Inc., USA) and imported into Image J software. The cell viability was calculated by dividing the number of dead cells by the total number of cells present.

5.2.3.5 Proliferation and Metabolic Activity

DNA quantification was performed to analyze the cell proliferation as described elsewhere [45, 46]. Briefly, DNA content on each scaffold was measured using the PicoGreends DNA quantitation kit (Invitrogen) at days 1, 7, and 14. The scaffolds (n=3 per group) were treated overnight at 60°C with 1 mL of DNA extraction solution (papain, Na₂HPO₄ and EDTA (Sigma-Aldrich). PicoGreen solution was then added to all samples and standards and incubated at room temperature for 10 min. The absorbance was
measured at 485 nm with a spectramax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) and the values were normalized to values taken from samples at day 1.

The metabolic activity of cells cultured on the scaffolds (n=4 per group) and well-plates as a control (n=3) were determined using the Alamar Blue (AB) assay (Invitrogen) according to the manufacturer’s protocol. Briefly, AB solution in DMEM at the ratio of 1:9 was added to each scaffolds and incubated for 4 h. Two hundred microliters of the AB solutions were then transferred to a 96-well plate in duplicate and the fluorescence was determined at an excitation/emission of 550/580 nm. During the incubation time, the viable cells metabolize the resazurin dye (blue) in AB solution to resorufin (pink). The measured values were normalized to values taken from samples at day 1.

5.2.3.6 Cell cytoskeletal organization

Immunostaining was performed on cell seeded scaffolds with aligned and random fibers to evaluate the cytoskeletal structure (F-actin) on day 14 of culture. After washing the samples in DPBS, the samples were fixed in 4% paraformaldehyde (PF) for 10 min at room temperature. The scaffolds were then soaked in 0.1% Triton X-100 in DPBS solution for half an hour to permeabilize the cells. Scaffolds were then blocked in 1% bovine serum albumin (BSA) for 1 h. F-actin staining was then performed using a 1/400 dilution of Alexa Fluor-594 phalloidin (Invitrogen) in 0.1% BSA. The cell nuclei were stained with DAPI, present in the mounting medium, added just before cover slipping.

5.2.3.7 Cell alignment analysis

We quantified the alignment of cells on aligned and random scaffolds by analyzing the shape of cell nuclei (stained with DAPI) on fluorescence images taken from 9 different locations on each scaffold. The shape of each nuclei was fitted to elliptical geometries in Image J software. The alignment angle was defined as the major elliptical axis with respect to the horizontal axis. The normalized alignment angles were grouped in 10 degree increments to compare the nuclei alignments within each aligned and random fiber condition.
5.2.3.8  \( \alpha SMA \) and Vimentin expression

The expression of \( \alpha SMA \) and Vimentin was visualized by immunostaining at day 14 of culture. The scaffolds were fixed with ice-cold methanol, rinsed and incubated for one hr with primary antibodies for mouse anti-human \( \alpha SMA \) (1:2000) (Sigma Aldrich Co., St. Louis, MO), or rabbit polyclonal anti-vimentin antibody (1:2000) (Abcam, Cambridge, MA) in 1% BSA in PBS. Scaffolds were then rinsed three times in DPBS, and incubated for 1 hr in species-specific fluorescein isothiocyanate (FITC) or Texas Red-conjugated secondary antibodies (1:300) (FITC or Texas Red anti-mouse IgG (Vector Laboratories, Burlingame, CA) in 1% BSA/DPBS. The cell nuclei were stained with DAPI, present in the mounting medium, added just before cover slipping.

5.2.3.9  PCR Analysis

RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was performed with an iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). DNase I (Invitrogen) digestion of RNA samples (0.5ug) was performed prior to reverse transcription.

**GAPDH**

Forward: 5’ AGCCACATCGCTCAGACAC’3

Reverse: 5’GCCCAATAACGACAAATCC’3

**COL1A1:**

Forward: 5’CCAAGAGGAGGGCCAAGAAGAAGG’3

Reverse: 5’GGGGCAGACGGGGCACACTC’3

**\( \alpha SMA \)**

Forward: 5’TATCAGGGGGCACCACCTATG’3

Reverse: 5’AGGAGCAGAAAAGTGGTTTAGA’3

All reactions were performed using Fast Star SYBR Green PCR Master Mix, at the default setting...
on an ABI Biosystems StepOne Plus Real Time PCR Machine with the following temperature profiles: denature at 95°C for 10 minutes, and 40 cycles of 95°C for 15 sec, and 60°C for 1 min.

5.3 RESULTS AND DISCUSSION

The application of directional electrospinning was used to fabricate scaffolds with aligned fibers which enhanced mechanical properties of anisotropy, stiffness and ultimate tensile stress. As the anisotropic mechanical characteristics of heart valves primarily rely on the microstructure of aligned collagen fibers existing in each layer of the heart valve leaflets [11, 181], it is necessary to study and understand cellular behavior and function on the engineered scaffolds, to assess the degree to which the anisotropic fibrous structure of native tissue is reproduced.

5.3.1 PGS:PCL Scaffold Structure and Mechanics

PGS has shown promising biological characteristics for supporting VICs yet its mechanical strength cannot withstand the pressures hold by the heart valve [46]. As a result, combining this material with another highly biocompatible synthetic polymer is a promising approach for engineering heart valve scaffolds [47]. Previously, our lab used electrospinning to develop PGS:PCL composite scaffolds with tunable stiffness. The process does not require post-processing (photocrosslinking or thermal curing) as opposed to other PGS blended fibrous scaffold fabrication techniques such as acrylated PGS [96, 255] and coaxial electrospinning using PGS/PLLA blends [256]. However, the fabricated constructs were minimally elastic (less than 5%) and non-anisotropic [47, 57]. In this study, we modified the fabrication technique to create PGS:PCL scaffolds with anisotropic characteristics and enhanced mechanical properties while maintaining cell functionality. It is expected that both the microstructure and mechanical properties of engineered scaffolds, resembling the native heart tissue, affect cellular behavior.

Furthermore, since the scaffold is prefabricated, the cells will not be exposed to the potentially toxic environments of scaffold fabrication. For example, the crosslinking of PEG-based scaffolds with
UV exposure or hydrogel-based materials with different chemicals or thermal conditions may induce cell death.

5.3.1.1 Fabrication and chemical characterization of microfibrous PGS:PCL scaffolds

Our composite materials contained PGS, which facilitated cell attachment and growth, and PCL, which provided mechanical support. Random or aligned fibers were fabricated with a different electrosprining collector apparatus (Fig. 38A). To align fibers, two parallel aluminum electrodes bordering a Teflon sheet collector were employed, whereas, random fibers were produced on an aluminum electrode plate. We fabricated electrospun PGS:PCL scaffolds at ratios of 2:1, 1:1, 1:2, and 1:4. The chemical characterization of these compositions, as well as PGS pre-polymer, and pure PCL were assessed using FTIR analysis (Fig. 38 B-C). Two primary bands were detected at 1726 cm\(^{-1}\) (stretching vibrations of the carboxyl (C=O)) and 1180 cm\(^{-1}\) (stretching vibrations of the ether groups (C-O)) in the PCL spectrum. Also it revealed other characteristics PCL bands such as C-O stretching vibrations at 1050 cm\(^{-1}\), symmetric C-H stretching at 2865 cm\(^{-1}\), and C-O and C-C stretching at 1296 cm\(^{-1}\) [257]. The PGS pre-polymer spectrum consisted of methylene groups (C-H) at 2933 cm\(^{-1}\), 2908 cm\(^{-1}\) and 1390 cm\(^{-1}\). The ester band was present at stretching vibration C-O bonds at 1173 cm\(^{-1}\), and the strong carbonyl stretching bonds (C=O) at 1732 cm\(^{-1}\) were also exhibited in the spectrum along with the weak absorption bonds at 930 cm\(^{-1}\) and 1300 cm\(^{-1}\) confirming the presence of aliphatic acid (sebacic acid) within the spectrum [258, 259]. All of the characteristic bands of PGS are overlapped with PCL bands with the exception of a peak at 1228 cm\(^{-1}\) ascribed to C-O groups. Though the different polymer compositions grant different mechanical features, described later, their chemical properties do not differ considerably even after brief immersion in NaOH to promote hydrophilicity and cell binding.

5.3.1.2 Physical Characteristics of PGS-PCL Scaffold

The porosity of bioengineered scaffolds has critical role in cell-cell and cell-matrix interactions. The presence of pores facilitates oxygen and media diffusion throughout the scaffold as well as providing an increased surface area for cell binding in 3 dimensions [260]. The pore sizes of aligned fiber scaffolds
and random fiber scaffolds were measured by SEM and compared for different combinations of PGS:PCL (Fig. 39 A-D). As the PGS:PCL ratio decreased, the pore size increased in the aligned fiber scaffolds (Fig. 39 A). The fabrication of aligned fibers with higher concentrations of PGS had lower viscosity and fewer polymer chain tangles. This surface tension affected the processing and resulted in beads and spindles [57, 261] and less aligned fibers in the 2:1 polymer ratio (Fig. 39A). In addition, there was a 2 fold increase in the aligned fibers’ size with increasing PCL concentration, that could be due to the larger relative molecular size of PCL compared to PGS (Fig. 39 D).

Engineered composite heart valve replacements should ideally provide temporary mechanical support while allowing for the generation of new ECM by the transplanted cells and host cells. To allow for the growth and remodeling of the fabricated valves by VICs and surrounding tissue, the scaffold must be degradable at predictable loss rates [37, 47]. The degradation of aligned fibrous scaffolds was studied as a function of bulk weight loss over a period of 10 days (Fig. 39 E). The degradation rate of the scaffolds was ratio dependent where scaffolds with higher PGS:PCL ratio were degraded faster. These observations are in agreement with confirm other reports where PCL had a resident time in vivo on the order of a few years whereas PGS degraded within weeks to months [184, 262, 263]. As shown in Fig. 39 E, the degradation rate (wet weight loss at 10 days) changed from 18% for the (1:4) ratio to about 40% for the (2:1) ratio. The scaffold wettability was also tested, and indicated that all ratios of fabricated PGS:PCL scaffolds were highly hydrophilic as water drops rapidly penetrated and spread through all tested compositions, enabling facile cell seeding but precluding contact angle measurement (data not shown).
Figure 39: **Scaffold fiber SEM characterization and degradation.** (A-B) SEM images of aligned and random fibrous scaffolds (scale bar: 100 μm). Pore size measurements demonstrated slightly higher pore sizes for aligned scaffolds with more PCL content. (C) The pores in random fibers were in the same size range. (D) Fiber sizes of aligned and random fibrous scaffolds were measured. Higher values were observed for the (1:4) PGS:PCL ratio aligned scaffolds compared to other ratios of aligned scaffolds. Similar trends were observed for the random fibers. (E) Representative weight loss curves of scaffolds following 10 days of soaking in PBS with 0.5M NaOH. As expected, scaffolds with higher PGS content degraded faster.

Normal leaflet opening and closure during blood flow are dependent on the anisotropic mechanical characteristics of the leaflet [37, 46]. Polymers with different properties spun in a defined manner, could be used for the replacement of these native valves if their mechanical features could be made to coincide. Here we used uniaxial tensile testing to characterize scaffold behavior under external stress. Representative stress-strain curves for all the ratios of the PGS:PCL scaffolds with random fibers
were compared (Fig.40A). Representative stress-strain curves for the 1:1 and 1:4 PGS:PCL scaffolds tested along (PD) or perpendicular (XD) to the direction of the aligned fibers were also compared (Fig.40B). The stress-strain curves for the random fibers exhibited a small linear region followed by a significant deformation known as creep (starting from approximately 8% strain). However, for aligned fibers, less deformation and creep were observed in the stress-strain curves similar to the trend for an elastic material. This could be due to the structural features of aligned fibers that are oriented along the direction of the tensile load [264]. Moreover, aligned scaffolds were stiffer in the PD direction compared to XD direction which resulted in anisotropic characteristics for these scaffolds. Interestingly, we observed a relation between the ultimate tensile strength and the thickness of scaffolds with aligned fibers as shown in Fig.40C (illustrated here with a ratio of (1:2)). While the initial stiffness of all three scaffolds with different thicknesses were similar, the ultimate tensile stresses decreased by increasing the thickness. The trend of the stress-strain curves for the thicker, aligned scaffolds resembled the same trend as scaffolds with random fibers with a large deformation region. This finding could be due to the fact that thicker scaffolds contain more random fibers due to the shielding of the polymer from the electrodes as the thickness of the scaffold increases (40μm average thickness with UTS of 6.44±1.70 MPa, 70 μm average thickness with UTS of 2.46±0.66 MPa and 140 μm average thickness with UTS of 1.47±0.15 MPa). As shown in Fig.40 D-F, the stiffness, UTS and anisotropic characteristics of scaffolds with aligned fibers improved (in PD direction) compared to the scaffolds with random fibers. The presence of more PCL content in the polymer solution increased the elastic modulus and ultimate strength with a lower strain to failure. Based on our results, electrospinning of PGS:PCL with the ratios ranging from (2:1) to (1:4) yielded scaffolds with anisotropic and tunable mechanical characteristics, which matched native tissue stiffness, ultimate tensile strength and anisotropy [46]. Mechanical properties (e.g. E, UTS and ef) of each of the different groups of scaffolds are listed in Table 3 in detail.
Figure 40: **Uniaxial mechanical testing of unseeded scaffolds in two directions.** (A) Representative stress-strain curves of random scaffolds for all the ratios of the PGS:PCL scaffolds demonstrated that scaffolds with random fibers deformed extensively following 5-10% strain. The initial stiffness and UTS increased by increasing the PCL content in polymer ratios. (B) Representative stress-strain curves of aligned scaffolds for (1:1) and (1:4) ratios of the PGS:PCL shown in two directions (PD and XD). Scaffolds were stiffer in PD directions. (C) Representative stress-strain curves of aligned scaffolds with different thicknesses depicted for the ratio of (2:1). Similar initial stiffnesses were observed with divergent relationships between UTS and scaffolds stiffness. As the thickness of the aligned scaffolds increased, greater deformations were observed in the stress-strain curves. This deformation was similar to that of the random fiber scaffolds (D-E). Tensile modulus and UTS were compared between random and aligned scaffolds in PD and XD direction. (F) Anisotropy of scaffolds was measured for PGS-PCL scaffolds of varying ratios, which demonstrated that the values for (1:1), (1:2) and (1:4) were similar to native tissues.
Table 3: Mechanical properties of aligned and random fibrous scaffolds

<table>
<thead>
<tr>
<th>Sample</th>
<th>Structure</th>
<th>Ultimate Tensile Strength (MPa)</th>
<th>Young’s Modulus (MPa)</th>
<th>Strain–to-failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2:1)</td>
<td>Random</td>
<td>0.73±0.26</td>
<td>2.09±0.91</td>
<td>2.04±0.78</td>
</tr>
<tr>
<td></td>
<td>PD</td>
<td>1.01 ±0.16</td>
<td>4.16±1.00</td>
<td>0.90 ±0.16</td>
</tr>
<tr>
<td></td>
<td>XD</td>
<td>0.48±0.14</td>
<td>0.95±0.68</td>
<td>1.67±1.01</td>
</tr>
<tr>
<td>(1:1)</td>
<td>Random</td>
<td>0.86±0.03</td>
<td>3.64±0.27</td>
<td>4.26±1.02</td>
</tr>
<tr>
<td></td>
<td>PD</td>
<td>1.5 ±0.55</td>
<td>6.31±1.59</td>
<td>0.8±0.29</td>
</tr>
<tr>
<td></td>
<td>XD</td>
<td>0.40 ±0.12</td>
<td>0.83±0.16</td>
<td>3.29±1.71</td>
</tr>
<tr>
<td>(1:2)</td>
<td>Random</td>
<td>1.3±0.18</td>
<td>5.32±1.45</td>
<td>2.86±0.96</td>
</tr>
<tr>
<td></td>
<td>PD</td>
<td>1.79 ±0.41</td>
<td>7.28±1.07</td>
<td>1.37±0.51</td>
</tr>
<tr>
<td></td>
<td>XD</td>
<td>0.77 ±0.09</td>
<td>0.6 ±0.19</td>
<td>2.57±0.4</td>
</tr>
<tr>
<td>(1:4)</td>
<td>Random</td>
<td>2.21±0.34</td>
<td>7.05±1.45</td>
<td>4.21±0.34</td>
</tr>
<tr>
<td></td>
<td>PD</td>
<td>3.98 ±1.18</td>
<td>9.28±0.43</td>
<td>1.15±0.22</td>
</tr>
<tr>
<td></td>
<td>XD</td>
<td>0.89 ±0.11</td>
<td>1.21±0.33</td>
<td>2.69±0.47</td>
</tr>
</tbody>
</table>

Fig. 41  A-C data include the mechanical properties of scaffolds in wet and dry conditions. Wetted scaffolds were less stiff and failed later, by UTS measurements, than dry scaffolds. In the case of (1:4) the ultimate tensile strength was particularly disparate between the wet and dry conditions. This effect could be attributed to the absorbed water decreasing resistance to deformation, whereas in dry conditions entangled fibers have a high sliding friction between polymer chains [265]. We found that the aligned patterns enhanced the elasticity and anisotropy of the engineered scaffold, and more closely resembled the native ECM architecture surrounding the VICs in vivo.

Figure 41: Mechanical properties of wet and dry scaffolds. Mechanical properties; (A) stiffness and (B) ultimate tensile strength and (C) anisotropy of scaffolds were measured in dry and wet conditions. Slight differences indicated for tensile modulus and UTS and no significant differences in anisotropy.
Fig. 42. demonstrated the effect of overall polymer concentration on scaffolds mechanical characteristic tested for 1:2 ratio of PGS:PCL.

Figure 42: Fiber mechanical properties; (A) tensile modulus and (B) UTS were measured for the ratio of (1:1) with different alignments. 0.20 (g/ml) Polymer concentration rendered scaffolds with higher stiffness and UTS.

5.3.2 Composites Developed with PGS:PCL Scaffolds Seeded with VICs

To assess the utility of PGS:PCL scaffolds for heart valve tissue engineering, we combined the electrospun fiber scaffolds with primary valvular cells and determined their reaction to a range of scaffold fiber stiffnesses and alignments. Though most research on TEHVs has been performed in vitro with porcine and other non-human cell sources, here we were able to use relevant human primary cells and directly compare them with non-human cell counterparts used in other studies [77].

Recently, more studies have been dedicated to TEHV scaffolds design [40, 45, 46], culturing tissues in bioreactors [49] and adopting appropriate cell types for clinical relevance. The complexity of heart valve physiology has received considerable attention particularly at the macro-scale to observe tissue behavior [37]. However, fewer studies have focused on the biosynthetic [65], regulation [229] and contractile [25] characteristics of interstitial cells [44]. This is significant given the recent interest in changes imparted to tissues through the stiffnesses of substrates. This is particularly evident in the field of tissue engineering, where artificial materials over a wide range of mechanical properties are combined...
with dissociated primary cells [266].

5.3.2.1 Cell Viability and Adhesion on Microfibrous PGS-PCL Scaffolds.

For the primary assessment of the cells interaction with scaffolds, cell attachment and viability were measured based on DAPI staining (Fig. 43A) and Live/Dead assays (Fig. 43B), respectively. The cells were distributed on the surface of the scaffold at a concentration of approximately 140-180 cells/mm² (Fig. 43C). Based on the initial cell density, these values indicated that 60-70% of the cells attached to the scaffolds. Cell seeding efficiency values were improved drastically over hydrophobic scaffolds that were untreated by NaOH immersion (data not shown). The results were promising while the differences of cell attachment between different combinations of PGS:PCL were statistically negligible. As was expected, since the cells were not exposed to lethal polymerization conditions and directly seeded on the pre-fabricated scaffold, the viability was high at 85% to 90% range. There was a slight decrease in cell viability during the 10 days of culture which is typical of cells seeded on synthetic scaffolds (Fig. 43D). Following on the encouraging viability results, cellular functionality on the scaffold was investigated.
Figure 43: **Human VIC Attachment and viability on aligned fibrous scaffolds.** (A-B) Representative images of DAPI (Nuclei: blue stain) and Live/dead staining of cultured VICs on the aligned scaffolds at day 1 (Live cells: green stain, Dead cells: red stain) (C) quantification of cell attachment at day 1 and (D) live/dead assay results at days 1 and 10 (scale bar =100μm).

### 5.3.2.2 Cell alignment analysis

There is evidence suggesting that collagen secretion and collagen fibril formation by VICs [26] is affected by transvalvular pressure and by the spatial arrangement of VICs across the circumferential direction in heart valve leaflets [267]. Previous studies have also confirmed the role of substrate topography[268, 269] on fiber alignment and cell spreading[270, 271]. As it is presented here, applying directional electrospinning not only enhanced the mechanical properties of PGS-PCL scaffolds but also aligned the attached cells toward the direction of polymer fibers (Fig. 44).
Figure 44: Human VIC alignment on fibrous scaffolds. (A-B) Actin filaments and nuclei stained with Phalloidin and DAPI on random and aligned scaffolds at day 14. Bottom panels are the quantitation of the alignment of VICs on the scaffolds. Cell alignment was significantly higher for the scaffolds with aligned fibers (~50-60% aligned cells toward the direction of fibers) (scale bar =100μm).

With the quantification of cell alignment, all randomly deposited PGS:PCL combinations were less than 20% aligned, whereas, aligned scaffolds were more than 50% aligned in the direction of the deposited fibers. Cell spreading improved on scaffolds with higher PGS concentrations at the earlier time.
points assayed (F-actin staining on scaffolds Fig.45), though the differences were minimal at later time points (alignment analysis comparison; Fig.44).

Figure 45: F-actin filaments and nuclei stained with Phalloidin and DAPI at day 1 for random scaffolds. Cells were initially more spread on scaffolds with more PGS (2:1) (scale bar =100μm).

The effect of substrate stiffness along with substrate structure on cell alignment is presented in Fig.46. Scaffolds that were less stiff (2:1 and 1:1 ratio) had slightly more aligned cells on the substrate. This result could be due to the lower resistance of the substrate to cell arrangement and matrix formation along the fiber direction [86]. These results suggested that the scaffold structure and to a lesser degree, mechanical characteristics have effects on the human VICs morphology and organization.

Figure 46: Quantified alignment comparison of human VIC-seeded scaffolds. Cell alignment was slightly higher on (2:1) and (1:1) PGS:PCL ratios. This finding can be related to the smaller fibers sizes in
composite with higher content of PGS. Also lower stiffness of the scaffolds affects the alignment in a way that cells aligned better on less stiff scaffolds.

5.3.2.3 Cell proliferation, metabolic activity analysis and protein expression

Besides mimicking an aligned fibrous scaffold with tunable stiffness, it is necessary to study the cellular behavior on the engineered structures. One of the distinct aspects of this study is the primary human VICs employed, which were evaluated for cellular functionality on PGS:PCL scaffolds with different mechanical characteristics. We studied the cellular proliferation, gene expression, and protein expression to compare the cellular responses to the different ratios of PGS:PCL scaffold produced.

Collagen Expression in human VICs cultured on Scaffolds:

In a study examining the stiffness at which VICs become activated, it was observed that VICs could be activated at stiffnesses above ~2.5KPa, (with or without the addition of TGFb), and in some settings, environmental stimuli (i.e. stiffness) had more of an influence on cell phenotype than chemical stimuli (i.e. TGFb) [272]. Fig.47 A depicts the collagen type I gene expression on cell seeded scaffolds, all of which were approximately one order of magnitude more stiff than those tested in the previous study [272]. We observed that more collagen was expressed by cells on the PGS:PCL composites than on the tissue culture plates, though at the ratios of (1:1) and (1:2) it was not statistically significant. The highest collagen expression occurred in cells on (2:1) PGS:PCL polymer ratios.

αSMA Expression in human VICs cultured on Scaffolds:

Other studies have shown that healthy adult VICs exhibit a quiescent, fibroblast-like phenotype, expressing vimentin and, to a lesser degree, αSMA (myofibroblast marker) [17]. During fetal development, active VICs are denser, proliferate more and undergo apoptosis more than in adult valves. After birth, these valve cells become more quiescent and the collagen content of the valves matures [273]. However, activated VICs have been observed in valves experiencing abnormal hemodynamic conditions
These activated VICs (myofibroblasts) begin proliferating [275] and contribute to excessive ECM remodeling, ultimately leading to fibrosis and disease [24, 276].

Similar to the diseased phenotype, in vitro culturing can activate the VICs to transform from a quiescent, αSMA negative phenotype, to an activated myofibroblast like phenotype with the properties of fibroblasts and smooth muscle cells, expressing αSMA and myosin with possible contraction [17, 24]. The αSMA positive phenotype has been observed in 50% to 78% of cells isolated from intact heart valves [24, 277]. This evidence has contributed to the consensus that culturing VICs on stiff 2D tissue culture polystyrene increases their activation and expression of αSMA, highlighting one of the multiple roles that substrate stiffness can play in modulating cell phenotype.

However, there has been limited research investigating the precise levels of stiffness required to induce myofibroblast activation. In a recent study, encapsulated porcine VICs in relatively stiff pure hyaluronic acid hydrogels spread more than in gels of lower stiffness. The cells also dramatically upregulated alpha smooth muscle actin expression, resembling activated myofibroblasts gel stiffness increased [278]. The few measurements have defined a substrate stiffness region of approximately 15kPa or more for VIC activation [279]. Here, human VICs cultured on our scaffolds had slightly higher αSMA expression for less stiff material (4 MPa for (2:1) to 9MPa for (1:4) ratio of PGS:PCL)(Fig.47). Moreover, lower expression of αSMA was observed on the scaffolds (about 1.5 fold) as compared to cells cultured on tissue culture treated plastic (stiffness ~3GPa [280]). Interestingly, we observed no changes in αSMA expression over time, similar to native valvular cells that remain quiescent and fibroblast-like (Fig.47 B).
Figure 47: The effect of aligned scaffolds on human VIC gene expression, proliferation and metabolic activity. (A-B) Collagen type I and αSMA expression, as measured by PCR, for human VICs seeded on PGS-PCL scaffolds measured on days 1 and 14. Cells cultured for 14 days on tissue culture plates were used as controls. (C) Cell proliferation based on total DNA content of human VICs on days 1, 4, 7 and 14. (D) Cell metabolic activity measured by Alamar blue assay at days 1, 7 and 14 for cells cultured on scaffolds and tissue culture plates (data has been normalized to initial values for comparison).

To further assay VIC phenotypic markers, cells on aligned scaffolds were immunostained for the presence of vimentin and αSMA proteins (Fig.48 A-B). Immunostaining was also performed for vimentin on the random fibers with no differences observed Fig.49.
Figure 48: The effect of aligned scaffolds on vimentin and αSMA expression. (A-B) Representative fluorescence images of human VICs immunostained for (A) vimentin (red), and DAPI (blue), and (B) αSMA (green) on the aligned scaffolds at day 14 (scale bar =100μm).

Figure 49: Vimentin expression at day 7 on random fibres seeded with human VICs. (scale bar =100μm).

Cell proliferation and metabolic activity:

Cell proliferation and metabolic activity were assayed on the different ratios of PGS:PCL scaffolds to evaluate the effect of substrate stiffness. The results showed that human VICs exhibited significantly enhanced proliferation and metabolic activity, about 3 fold and 2 fold respectively, following 14 days of culture compared to days 1 and 7 (Fig.47C-D). Contrary to what was observed with porcine cells (Fig. 50), human cells had a slower rate of proliferation and lower metabolic activity in the initial week of culture. The human cells required more time to initially adapt to their environment before regular
proliferation commenced. There was not a considerable difference between each ratio of the PGS:PCL scaffolds as expected and this is likely due to the presence of PGS in the composite. Previously it has been shown that any combinations of PGS:PCL scaffolds have higher cellular metabolic activity than pure PCL scaffolds [57]. Here, the cultured cells on scaffolds were shown to have a favorable tolerance of each of the PGS:PCL ratios tested.

5.3.3 Porcine VICs Response to PGS:PCL Scaffolds

Fig. 50 is comprised of the data with porcine VICs cultured on PGS:PCL scaffolds for 10 days. At day 1 cells spread more on the less stiff (less PCL content) constructs (Fig. 50 A), as demonstrated for the (2:1) and (1:4) ratios. This difference was also observed for the human VICs wherein the cells were spread more at day 1 on less stiff scaffolds (Fig. 45). These analyses were made by staining the cells on the scaffolds with F-actin. We did not observe large changes in cell spreading over time on scaffolds (Fig. 50).

Cell attachment, viability, proliferation, and metabolic activity were also assessed for the porcine cells. There were no significant differences between the ratios for all of the above analytics, similar to the human VIC results. Cell attachment was slightly higher for the porcine VICs (about 85% of the initial cell seeding density) when compared with human VICs. Human VICs also differed in their time needed to acclimate to the polymers. Freshly seeded human VICs initially underwent a stationary/lag phase, whereas porcine VICs began proliferation and metabolic activity as soon as 3 days after seeding (Fig. 50 E-F).
Figure 50: Characterization of porcine VICs on random PGS-PCL scaffolds. (A-B) Fluorescence microscopy images of porcine VICs stained with Phalloidin and DAPI to stain actin and nucleous respectively. (A) Cell morphology was compared for ratios of 2:1 and 1:4 at day 1 and more spreading was observed for scaffolds with more PGS content and less stiffness. (B) Cell morphology of porcine VICs are shown for ratios of (1:1) and (1:2) for days 1 and 14. Porcine VICs quantified for (C) cell attachment, (D) viability, (E) metabolic activity, and (F) proliferation.

5.4 CONCLUSION

Here we have taken a tunable polymer composite and molded it to form and meet the functional demands of heart valve replacement therapies. We developed microfibrous PGS:PCL scaffolds, including random and aligned fibers that support human VIC engraftment and maintenance in vitro. The mechanical properties and anisotropic characteristics of fabricated scaffolds were related to the ratio of PGS:PCL content in the scaffolds. Both human and porcine VICs were successfully cultivated for approximately 2 weeks to evaluate the cellular behavior on the fabricated composites. We demonstrated that scaffold structure affects the cellular arrangement considerably, whereas the substrate stiffness (in the range of 4-9 MPa) had minimal effect on the cell alignment and was supportive across the range tested. VIC
attachment, viability and proliferation followed similar trends for each polymer ratio while there was a considerable difference between the results obtained with human and porcine cells. Scaffolds with a (2:1) ratio of PGS:PCL expressed higher levels of collagen I, while αSMA expression was slightly higher for less stiff scaffolds. Interestingly, αSMA expression on scaffolds was considerably lower compared to those in plated cells which indicated the ability of the scaffolds to maintain the cells in a more quiescent condition similar to native tissues. Lack of porosity, elastic deformation and appropriate thickness will be considered in future work on these constructed composite scaffolds. Therefore, ongoing studies will be devoted to manipulation of the constructed scaffolds to address these limitations with the aim of further promoting engineered heart valve tissue formation and eventually providing viable heart valve replacement options.
6 MSCS SEEDED ELASTOMERIC TRI-LAYERED SCAFFOLDS TO MIMIC HEART VALVE LEAFLET’S STRUCTURE AND MECHANICAL PROPERTIES

6.1 INTRODUCTION & RATIONAL

As described there are several limitations associated with currently available heart valve prostheses. Mechanical valves are thrombogenic and require patients to undergo lifelong anticoagulation therapies [36, 37, 39, 281]. Bioprosthetic heart valves have limited durability because of their susceptibility to degradation and calcification [37, 38]. These drawbacks are particularly apparent in pediatric valvular diseases due to the inability of the graft to grow in vivo with the patients, requiring subsequent operations. Autologous TEHV aim to overcome these limitations by creating living, biocompatible, mechanically analogous heart valve replacements able to grow and remodel with the patient [44, 52, 53, 58, 282-284].

Heart valves primarily consist of VICs and are surrounded by an endothelial monolayer [285]. The ECM of heart valve leaflets is a complex three-dimensional (3D) tissue, consisting of three interconnected layers; the zona fibrosa, the zona spongiosa and the zona ventricularis, primarily composed of collagen, glycosaminoglycans (GAGs) and elastin respectively [285]. This intricate ECM layered architecture and its anisotropic structure determine the mechanical characteristics of valve leaflets [9, 11, 181, 251]. More specifically, leaflet mechanical stiffness and elasticity are attributed to the surface fibrous layers; zona fibrosa and zona ventricularis [32]. Considering that valve function is dependent on its structure, ideally, engineered scaffolds should (a) resemble the native tissue microstructure [250, 251], (b) match native mechanical properties and retain anisotropy [11, 252], (c) have elastic characteristics in deformation similar to native tissue, and finally (d) possess a controlled degradation rate that preserves structural integrity while providing support for adequate tissue formation [47].

A typical TEHV approach is to seed natural [40, 41]or synthetic biocompatible scaffolds with cells [42-48], culture them in static in vitro environments or in bioreactors simulating tissue growth with physiological hemodynamics [49-51], and then implant the cell-seeded constructs in vivo [44, 52, 53].
Cell sources such as vascular-derived smooth muscle cells [51], MSC [59], VICs [26, 46, 47], and fibroblasts [45], have been utilized to engineer functional TEHV. In particular, studies have aimed to create scaffolds, mimicking the structural complexity of heart valves while providing an appropriate environment for cellular growth and ECM generation in vitro [40, 42, 44-46, 52]. However, some of the drawbacks associated with these engineered scaffolds have prevented them from successful translation to in vivo models. These limitations include unnaturally large stiffnesses (e.g. non-woven scaffolds) [44, 50, 55], lack of anisotropic characteristics (e.g. homogenous fibrin gels or fibrous scaffolds comprising of random fibers) [41, 42, 47, 58], non-fibrous structures with large pores [43, 45, 46], non-physiological deformation [47, 57] and lack of suturibility (e.g. hydrogel based scaffolds and microfabricated scaffolds) [40, 286]. Biodegradable elastomers have been synthesized for cardiac tissue engineering for their potential to withstand physiological cyclic loads and viscoelastic properties resembling native tissues [145, 287-289]. Among these, PGS has been employed extensively due to its elasticity, biocompatibility and controlled degradation rate [45, 46, 55, 184, 248]. However, the modulus of PGS varies between 0.18 MPa to 1.5 MPa depending on curing conditions (time and temperature) and scaffold structure [45, 72, 183], which is considerably lower than native leaflet stiffness (4-8MPa) [32, 46].

We have previously shown that fibroblast and VIC seeded microfabricated PGS scaffolds, with diamond shape pores, provided adequate anisotropy in accordance with native leaflet properties while supporting tissue formation and ECM deposition [45, 46]. However, the mechanical characteristics of the seeded constructs did not match the native leaflet stiffness precisely. In addition, the microfabricated PGS scaffolds lacked fibrous structure and the existence of micropores limited their suturability, impeding their application for in vivo studies. In comparison, PGS/PCL electrospun scaffolds had tunable mechanical properties, which adequately matched the stiffness of native heart valve leaflets [57]. However, random fibers structure failed to offer anisotropic mechanical characteristic, similar to native tissue properties. Also, electrospun PGS/PCL scaffolds experienced a large creep deformation associated with their stress-strain curves. In addition, the presence of small pores in the structure of these scaffolds
(pore size <8μm) prevented cell migration and ECM deposition through their 3D structures, which limited the formation of a 3D tissue construct. To improve our existing design and address current limitations, the present study has aimed to develop a novel tri-layered scaffold with elastomeric and anisotropic properties inspired by the structure and mechanics of native leaflets. A semi-automatic layer-by-layer assembly was applied to fabricate the biocompatible and mechanically tunable 3D construct. We hypothesized that combining PGS/PCL microfibers and microfabricated PGS in a tri-layered construct provided elastic and anisotropic characteristics for the 3D architecture which matched structural and mechanical properties of native leaflets while simultaneously supporting controlled cellular growth and tissue formation within a guided architecture. This approach could have the potential for successful translation towards a tissue engineered heart valve replacement.

6.2 MATERIALS AND METHODS

PGS microfabricated scaffolds were designed and obtained through a micromolding procedure described in detail previously [46]. Following scaffold fabrication, directional electrospinning was applied to create aligned PGS/PCL fibers on the microfabricated scaffolds between two parallel aluminum plates as previously explained in Chapter 5 [290]. The scaffolds were tested with an uniaxial mechanical tester to assess the mechanical characteristics of the unseeded scaffolds initially and after 4 weeks culture period (soaked in medium). The samples were then steriley prepared for cell seeding by soaking in media for 2 days. Two cell seeding techniques, including rotary and static seeding procedures were applied in sequence to increase cell attachment. At each time point, samples were frozen and stored for biochemical assays, including collagen, GAG and DNA assays to assess the tissue formation and cellular proliferation. Samples were fixed and cut for histology and immunohistochemistry. The designed tri-layered scaffolds were implanted as a single PV leaflet in fresh porcine hearts. The hearts were connected to sets of tubing to apply flow on the pulmonary side with hydrostatic pressures in accordance with physiological values. The opening and closing of the scaffolds was visualized and compared with the single fibrous layer scaffolds.
6.2.1 Fabrication of Tri-layered Scaffold Containing Microfabricated PGS and PGS/CPL Fibers

*Polymer synthesis:* PGS pre-polymer was synthesized through polycondensation of glycerol and sebacic acid (1:1 molar ratio) by adapting the methods of Wang *et al.* [145] In brief, sebacic acid and glycerol with 1:1 molar ratio were reacted at 120°C in high vacuum (~6.5 Pa) for 24 hr to synthesize PGS pre-polymer.

*Micromolding Fabrication:* The fabrication process used to design the PGS scaffolds, consisting of 2:1 aspect ratio diamond shape pores with approximately 75μm-thick struts, was previously described in detail [46]. The mold was made from an ultra-high temperature machinable glass-mica ceramic sheet (0.5" thick, 2” x 2”, McMaster-Carr, Elmhurst, IL). The design was cut through the ceramic sheets using a dicing cutter machine (Kulicke & Soffa Industries, Inc.,Fort Washington, PA) with a 90 μm wide saw blade. PGS pre-polymer was then melted around the edges of the ceramic mold and allowed to flow into the channels of the fabricated mold. The PGS pre-polymer was then cured in a vacuum oven under high vacuum (< 6.5 Pa) at 160°C for 8 hr. A razor blade was used to cut the ceramic fabricated part away from the bottom sheet and release the polymer scaffold from the ceramic sheet yielding a 300 μm thick porous scaffold. Prior to the electrospinning process, scaffolds were treated with oxygen plasma cleaner to improve adhesion of fibers on the scaffold layers (100 W for 30 s for each sides using Harrick Plasma (Ithaca, NY)).

*Electrospinning:* A directional electrospinning system was employed to spin the pre-polymers into sheets as described in Chapter 5. The pre-polymer mixture was pushed from a syringe pump at a flow rate of 2 mL/hr and 18 kV. The distance from the tip of the gauge needle to the microfabricated scaffolds was set at 18 cm. The microfabricated PGS was placed between two aluminum electrodes (separated by 1.5 cm) and aligned fibers were created between two grounded electrodes for approximately 15 min on either side. The tri-layered scaffolds were then desiccated in a vacuum chamber overnight for solvent evaporation before further characterization.
6.2.2 Sheep MSCs and VICs Isolation and Scaffold Cell Seeding

Valve Leaflet Dissection and VICs/MSCs Isolation: Leaflets were aseptically excised from fresh sheep hearts, obtained from the Animal Research Facility (ARCH) in Childrens’ Hospital (Boston, MA) under an official review panel approved protocol. Individual leaflets were excised and rinsed thoroughly in a 2% (v/v) solution of antibiotic-antimycotic in Hank’s Balanced Salt Solution (HBSS, Invitrogen Carlsbad, CA) to remove any remaining blood cells. The samples were cut in circumferential and radial directions for further mechanical characterization. The remaining tissues were used for VIC isolation or frozen for biochemical assays.

Bone marrow samples were obtained from sheep femurs in ARCH. Prior to the isolation process the samples were preserved in isolation buffer (ACD solution and heparin sulfate (American Pharmaceutical Partners)) on ice. 15 ml of Ficoll-Paque Plus (Amersham Pharmacia) was added to each 50 ml Accuspin tube (Sigma-Aldrich, A2055) and spun for 1 min (1200 rpm) to sediment the Ficoll-Paque. The mononuclear cell layer was collected with a syringe and transferred into 50 ml conical tubes on ice. Every 10 ml of collected cells were mixed with 5 ml isolation buffer. The cell pellet was obtained following two sequential spinning and resuspension cycles in isolation buffer. The cells were then ready for cultivation and further harvest.

Pulmonary VICs were isolated as described previously [46] by collagenase digestion. Briefly, the leaflets were wiped with a sterile gauze to remove the valvular endothelial cells and VICs were isolated through digestion of the leaflet tissue in a solution of 0.5% (w/v) type I collagenase (Worthington Biochemical) in HBSS at 37°C for 6 hr. The digested tissue was then centrifuged at 1000 g for 10 min and the isolated cells were then resuspended and expanded in culture medium of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic (pen/strep).

Cell Seeding: In preparation of cell seeding, scaffolds were first sterilized by soaking in 70% ethanol for 30 min followed by high intensity UV exposure (800 mW) for 5 min on each side. The
scaffolds were then soaked in culture medium for 2 days prior to seeding to improve cell attachment. Each scaffold was then placed into a sterile-vented 50 ml bioreactor tube (TPP Techno Plastic Products AG, Trasadingen, Switzerland). Confluent flasks of sheep pulmonary VICs and MSCs were trypsinized (0.25% (w/v) trypsin, 1 mM EDTA; Invitrogen) and resuspended in culture medium such that 8 ml of cell solution was added to each 50 ml tube resulting in a cell density of approximately 1x10^6 cells/cm^2. The tubes were then rotated for 24 hr inside an incubator at 37°C and 5% CO_2. After cell attachment, the scaffolds were placed in to individual wells of a non-adhesive 6 well plate (Costar Ultra Low Attachment; Coming, NY) and were subjected to static cell seeding by applying approximately 1x10^6 cells/250 µl media on either side of each scaffold.

6.2.3 Uniaxial Mechanical Testing

Native tissues, single and tri-layered scaffolds were tested by uniaxial mechanical Instron machine (Model 5542, Norwood, MA) to characterize the scaffolds and tissues mechanical properties. Samples were cut into 15 mm by 5 mm rectangular strips. Geometry data were imported in the Blue Hill mechanical testing software and samples were stretched to failure using a 10 N load cell to measure the reaction force. The samples were loaded at a 7 mm/min extension rate.

For the native PV and AV tissues, we measured the initial modulus (0-15% strain region; equivalent to the Young’s modulus for a linear elastic material for scaffolds), transient modulus and the peak tangent modulus (i.e., the modulus in the steepest region of the stress-strain curve). The ultimate tensile strength (UTS) and the strain-to-failure for native tissues and microfabricated PGS were measured. Yield stress (Yσ) and yield strain (Yε) were obtained for fibrous and tri-layered scaffolds (to ignore the plastic deformed region).

6.2.4 Extracellular Matrix Generation and Cell Proliferation on Scaffolds

Samples (~2.5 by 2.5 mm) were cut from the cell-seeded scaffolds and weighed prior to the extraction of the ECM. The Sircol™ collagen assay kit (Biocolor L'Td., United Kingdom) was used as per
the manufacturer’s protocol to quantify the collagen content that was synthesized following the 2 and 4 weeks cultivation. In order to extract the collagen, samples were placed in PCR tubes in 100 μL of extraction solution (0.5 M acetic acid and 1 mg/ml pepsin A in water) overnight on an orbital rocker at room temperature. GAGs were extracted utilizing the Sircol™ GAG assay kit (Biocolor LTd., United Kingdom). Briefly, the samples were soaked in a 1 ml solution of 4 M guanidine-HCl and 0.5 M sodium acetate overnight at 2-8°C. Following the extraction steps, ECM proteins (collagen and GAG content) were measured according to the protocol provided with the Sircol™ assay kits using a Genesys 20 spectrophotometer (Thermo Spectronic, Rochester, NY).

DNA content was quantified on fibrous, microfabricated and tri-layered scaffolds at each specific time point by using a PicoGreen dsDNA quantification kit (Invitrogen) per manufacturer’s instructions using a Spectramax Gemini XS plate reader (Molecular Devices, Inc., Sunnyvale, CA). Samples (~2 mm by 2 mm) were first cut from the cell-seeded scaffolds and weighed. The samples were then incubated in microcentrifuge tubes with 1 ml of buffered 0.125 mg/ml papain solution (DNA extraction solution) for 16 hr in a 60°C water bath before performing the PicoGreen assay.

6.2.5 Histology and Immunohistochemistry

Samples were first fixed in 4% PFA for 30 min, then rinsed in PBS, after which they were stored in 30% sucrose solution at 4°C overnight. Then samples were rinsed with PBS and embedded in OCT (Finetek). Cryosections of 10 µm were cut and stored at -20°C. Sections were thawed for 30 min before performing hematoxylin and eosin (H&E) staining for general morphology. To visualize myofibroblast-like differentiation, cell-seeded scaffold sections were stained for alpha smooth muscle actin (a-SMA, mouse monoclonal 1A4, Dako) using immunofluorescence. Normal horse serum (4%) was used as blocking solution. AlexaFluor 488 labeled secondary goat-anti mouse (Invitrogen) served as the secondary antibody. Sections were coverslipped with DAPI-containing Vectashield mounting media to counterstain the nuclei. Images were taken with a Nikon iEclipse microscope equipped with a digital camera (Nikon Instruments, Melville, NY).
The cell-seeded scaffolds were prepared for nuclei and F-actin visualization. Samples were first rinsed in HBSS and then fixed in 10% neutral buffered formalin (Sigma) for 20 min. The samples were then allowed to incubate at room temperature for 2 hr in 0.2% (v/v) Triton X-100 (Sigma) in Hank's Balanced Salt Solution (HBSS). The samples were then rinsed 3 times for 5 min each in 0.05% (v/v) Triton X-100 in HBSS and then blocked in 1% (w/v) bovine serum albumin (Sigma) and 0.05% (v/v) Triton X-100 in HBSS for 2 hr. Once the blocking was complete, samples were incubated for 3 hr in Alexa Fluor 488-phalloidin (1:40 (v/v) dilution of stock solution in 1% (w/v) bovine serum albumin and 0.05% (v/v) Triton X-100 in HBSS; Invitrogen). The scaffolds were then rinsed 5 times for 5 min each in HBBS and stored in the refrigerator overnight. The samples were then placed on glass slides and cover slipped with a drop of Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) to counterstain cell nuclei.

6.2.6 Thrombogenicity Assay

Human platelet rich plasma concentrates with approximately 1,000,000 platelets/ml was obtained from (ZenBio, Inc. NC). The platelets were spun down in 50 ml tubes (2700 rpm for 5 min). The pellet was resuspended in 500 µl of media which led to a concentration of about 100,000,000 platelets/ml. Scaffolds were washed with PBS and placed in 12 well plates. Samples were submerged in 400 µl of the platelet solution for 1 hr on a rocker in an incubator. Following the soaking process, samples were washed with PBS, fixed in 10% formalin for 20 min and immunohistology was conducted as described above using anti-human CD41 (Invitrogen Carlsbad, CA) (1:200 for 1 hr at 37°C) as a primary and anti-mouse Alexa568 (1:40 for 1 hr at room temperature) as a secondary antibody. Samples were stained with mouse anti-human CD41 (Invitrogen, Carlsbad, CA) (1:200 for 1 hr at room temperature). The samples were then washed and soaked in a solution of alexaflour 568 antimouse (1:40 for 1 hr at room temperature).

6.2.7 Microscopy

*Scanning electron microscopy (SEM) and Confocal Microscopy:* Scaffolds were imaged at different magnifications (e.g., 50x, 100x) using an environmental scanning electron microscope (ESEM),
SEMXL30 at low vacuum with a 32 kV accelerating voltage, 11 mm working distance. Immunohistology was visualized using a fluorescence microscope equipped with florescence camera (Axio Cam. MRm) and manufactured ApoTome for depth imaging (Carl Zeiss MicroImaging, Gottingen, Germany).

6.2.8 Engineered Heart Valve Single Fibrous Layer and Tri-layered Scaffold Functionality Test in an Ex vivo Experiment

An ex vivo experiment was designed to test the scaffolds as a single leaflet replacement for PV. Fresh porcine hearts were obtained from a local abattoir and the right ventricles were cannulated and connected to a water reservoir. The position of the bag was set at an exact height providing a hydrostatic pressure similar to the systolic blood flow pressure at the position of the PV (about 30 mmHg). The pulmonary artery was connected to a second water reservoir through a tube, which provided 10 mmHg of pressure during diastole. The repetitive cycles of systole and diastole were manually controlled by opening and closing clamps (attached to the tubing system) while the implant was visible during each cycle in real time. The scaffolds opening and closing was visualized using a surgical endoscope cannulated through the ventricles right beneath the PV position. Both single layered fibrous scaffold and tri-layered scaffold were examined as PV single leaflet implants. The tri-layered scaffolds were sutured through the top and bottom fibrous layers to the pulmonary artery in accordance with the native structure where the fibrosa and ventricularis layers are holding the scaffolds to the sinus wall.

Statistical Analysis:

Data is reported as mean ± standard deviation (SD). For group comparisons, one-way ANOVA followed by Tukey's post hoc tests were conducted. P values < 0.05 were regarded to indicate significance (*: P<0.05; **: P<0.01 and ***: P<0.001).
6.3 RESULTS & DISCUSSION

6.3.1 Scaffold Design and ESEM

Tri-layered scaffolds, resembling native leaflet tissue layers were designed using micromolding and electrospinning techniques. Our fabrication approach enabled us to combine the respective strengths of the separate materials PGS and PCL. The low molecular weight of PGS impairs the electrospinning of pure PGS to create a fibrous structure. In comparison, PCL demonstrates higher mechanical stiffness and facile electrospinning fabrication, but provides a less convenient environment for tissue cultures.[47, 291] Therefore, mixing these polymers may be resulted in the formation of a highly biocompatible structure with mechanical strength analogous to its native counterpart. Here we applied a procedure to assemble the 3D construct with tunable architecture. Microfabricated PGS layer was first fabricated using micromolding technique as described in Chapter 3 (Fig.51 Ai) [46]. This casting process resulted in the formation of PGS scaffold with anisotropic properties as a consequence of the unique diamond pore geometries as shown by environmental scanning electron microscopy (ESEM) images in Fig.51 B-C. Following PGS microfabrication, a directional electrospinning technique was used to generate aligned PGS-PCL fibers on either side of the microfabricated PGS scaffolds (Fig. 51 Aii), resembling the layered structure of native leaflet (Fig.51 Aiii). ESEM images from the cross-section of the tri-layered scaffold and the aligned fiber sheet with anisotropic characteristics, covering the microfabricated PGS layer are depicted in Fig.51 D-E, respectively. Note that preferred direction is parallel to the diamond long axis (PD) and XD is orthogonal direction. As demonstrated by ESEM images (Fig.51 F-G), fibers provided homogeneous interpenetrating pores in the structure of microfabricated PGS. This aligned network enhanced structural integrity while preserving the anisotropic characteristic of the microfabricated PGS scaffolds. Various layer-by-layer assembly methods have been previously used to generate 3D porous structures, which depend on manual stacking or require complicated technologies to control the bonding procedure [46, 75, 84, 292]. We applied directional electrospinning between two parallel electrodes[290] to cover both sides of microfabricated PGS layer with aligned PGS/PCL fibers. This process eliminated
the use of complex stacking or bonding technologies. In addition, we were able to achieve strong bonding between the layers by electrospinning directly on the plasma-treated microfabricated PGS scaffolds,[84] which overcome layer delamination issues, mainly associated by layer-by-layer assembly techniques.

Figure 51: **Schematic diagram of scaffolds fabrication process and SEM micrographs.** (A) Schematic demonstrates the fabrication of microfabricated PGS scaffolds. PGS solution was first poured on a ceramic mold containing diamond patterns and cured at 160°C for 8 hr. The surface layer was then delaminated from the mold and rinsed with PBS to remove the ceramic pieces from the microfabricated scaffolds. To form the tri-layered composite scaffold, the PGS sheet was then placed between two aluminum electrodes, connected to a ground. PGS/PCL fibers were electrospun on either side of the PGS layer. SEM micrographs of microfabricated scaffolds (B) without and (C) with a layer of aligned fibers underneath. (D) Aligned fibers of PGS/PCL scaffolds and (D) Cross section of tri-layered scaffolds comprised of a PGS layer and fibers on either surface. (F) Aligned fibers on top and (G) bottom of the diamond pore (scale bars: 100 μm).

### 6.3.2 Mechanical Properties of Unseeded Scaffolds with Degradation Study

In order to support cellular proliferation and adequate ECM formation by the cultured or host cells, the tissue engineered construct should ideally sustain mechanical support, while facilitating tissue growth during controlled scaffold degradation [11, 47]. The influence of the degradation process on the
Mechanical properties of scaffolds, designed for cardiac tissue engineering, have been widely discussed [46-48, 72]. This is an important factor to be considered in clinically translational TEHV technologies. The scaffold’s degradation and mechanical properties were assessed by incubating unseeded scaffolds for 4 weeks in normal growth media at 37°C (Dulbecco's Modified Eagle Medium (DMEM)). Initial stiffness (tensile modulus; E), yield stress (Yσ) and strain to deformation representing yield stress (Yε) were measured at time zero and following 2 and 4 weeks cultivation for single layer (layers (PGS/PCL electrospun sheet and microfabricated PGS sheet) and tri-layered scaffolds (Figure 52A-C). Lower stiffness and strength corresponding with higher elasticity (elongation) of the scaffolds are an indication of degradation and loss of mechanical strength (for tri-layered construct; stiffness, Einit=3.01±0.58 Vs. E4wk=1.63±0.36 MPa, (P<0.001) Yεinit=0.61±0.08 Vs. Yε 4wk=0.93±0.15 (P<0.001)). The highest rate of degradation was obtained with the microfabricated scaffolds and the lowest for the fibrous scaffolds (45% loss of stiffness for PGS scaffolds vs 20% stiffness reduction for PGS/PCL fibrous scaffolds). These results are in agreement with reported data from recent studies where PCL had a resident time in vivo in the order of 2-4 years whereas for PGS, it was weeks to months [184, 262, 263].

Figure 52: Uniaxial mechanical testing of unseeded scaffolds. (A-C) Uniaxial tensile mechanical properties of unseeded scaffolds in PD directions, tested following 4 weeks soaking in the media, demonstrating that (A) E and (B) UTS decreased whereas (C) ef increased for degrading scaffolds with time(*: P<0.05; **: P<0.01 and ***: P<0.001).

Cyclic tensile testing was performed to evaluate the reversible deformation and elastic characteristics of the tri-layered scaffolds compared to native tissue leaflets (Fig.53). The energy loss of
the fabricated tri-layered scaffolds was found to be 32.18±1.95 MJ/m³ following cyclic tensile loading up to 30% strain (corresponding with native tissue strain amplitude during diastole), which was similar to that leaflets native tissue (27.22±3.55 MJ/m³).

![Graph](image)

Figure 53: Representative cyclic stress-strain curves for tri-layered composite scaffold and native tissue are presented to compare the energy loss during 4 repetitive cyclic tests.

Representative stress-strain curves for fibrous PGS/PCL, microfabricated PGS scaffolds and tri-layered composites were depicted and compared to each other (Fig.54 A). Generally, for a material that experiences creep deformation following a linear trend in the stress-strain curve, the yield stress would be the point that defines the strain amplitude, in which the material deforms permanently and loses its deformation recovery ability [9, 32]. Due to the presence of PCL, a highly plastic material, tri-layered scaffolds and fibrous scaffolds underwent a slight deformation up to a certain strain, known as yield point [57]. Therefore, it is essential to consider only the linear portion of the stress-strain curve for those scaffolds that were able to recover during the stresses of normal blood circulation. Consequently, for those scaffolds containing PCL, Yσ and Yε were obtained as shown in Fig.54 A in addition to initial tangent modulus (E). However, since PGS is an elastic material with linear stress-strain curves [72, 145] the mechanical properties for microfabricated PGS scaffolds represented the initial tangent modulus (E), ultimate tensile strength (UTS) and strain to failure (εf). Representative stress-strain curves for tri-layered
composite containing aligned fibers and microfabricated PGS, were depicted in both PD and XD directions and confirmed the anisotropic characteristic of the designed constructs (Fig.54B).

![Graphs showing stress-strain curves](image)

Figure 54: **Representative stress-strain curves of fibrous, microfabricated PGS scaffold, and tri-layered composite scaffold in the PD direction.** (A) We observed a creep deformation in fibrous and tri-layered scaffolds following 25-50% strain (slightly less for tri-layered scaffold) while PGS showed a completely elastic behavior with linear trend in stress-strain curve (the initial stiffness E, Yσ and Yε were obtained as shown in graphs). (B) Representative stress-strain curves of tri-layered scaffolds presented in PD and XD directions, indicating the anisotropic properties of the construct. * represent the yield points for trilayered and microfibrous scaffolds and breaking point for PGS microfabricated scaffolds. Data corresponding to this point were measured and depicted in the graphs.

### 6.3.3 Scaffold Anisotropy and Suture Retention Characteristics

Normal leaflet opening and closing during blood circulation are dependent on the anisotropic mechanical characteristics of the leaflet [37, 46]. We measured the anisotropic characteristics of the engineered composite and compared the results with the values obtained for native PV and AV (Fig.55A). Although the tri-layered scaffolds were highly anisotropic compared to existing TEHV constructs, the stiffness anisotropy obtained for these scaffolds was considerably lower than native PV values. This could be due to the presence of dense collagen networks in AV and PV. However, the strength anisotropy of the fabricated tri-layered scaffolds was similar to the native tissue UTS anisotropy. A suture retention test
was performed on both native tissue (pulmonary artery (PA)) and fibrous scaffolds and similar results were obtained for the sutured scaffolds and native tissues (Fig. 56 A-B) where the sutured material ruptured at 0.84± 0.15 MPa while the native tissue ruptured at 0.46±0.21MPa.

Figure 55: Comparison between the scaffold anisotropy with native tissue, demonstrating that while the UTS anisotropy of the scaffolds was similar to native tissue values, the E anisotropy was considerably lower than that of native tissues.

Figure 56: (A-B) Suture retention tests on fabricated scaffolds and native tissues, demonstrated comparable results.
6.3.4 Mechanical Properties of Cell Seeded Tri-layered Scaffolds VS Native Tissue Mechanical Characteristic

Stress-strain trend analysis indicated the structural differences between the designed tri-layered scaffolds and native tissues (FIG. 57 A-B). Due to the crimped structure of collagen fibers in the native leaflets (both pulmonary valve (PV) and aortic valve (AV)) (FIG. 57 C-D), less force was required initially to stretch the leaflets up to the point where the collagen fibers were straitening (up to approximately 15-20% strain). This part is known as the initial region of the stress-strain curve. Following the transient region, where the fibers were completely straight, higher force was required to further deform and stretch the leaflets, which resulted in a sudden increase in the slope of the stress-strain curve and a steeper trend of the stress-strain curve in the peak region (FIG. 57 C,D). The mechanical properties and stress-strain trend of sheep valve leaflets are similar to what obtained for porcine samples as described in Chapter 4. Higher stiffness of the aortic leaflet is associated with the higher pressure in the aortic sides. Ultimate tensile strength and strain to failure were also obtained from experiments and data are presented in (FIG. 57 E-H), in both circumferential and radial direction. As obtained from stress-strain curves, leaflets are more deformable in radial direction with higher strength in circumferential direction.
Figure 57: Mechanical properties of AV and PV. Native tissue specimens were cut from sheep explanted leaflets in (A) circumferential (CIRC.) and (B) radial (RAD.) directions. (C, D) Representative stress-strain curves of native aortic and pulmonary leaflets illustrate the nonlinear, anisotropic mechanical response of the (C) AV and (D) PV. (E-F) Tangent moduli E were calculated for AV/PV in three regions and presented as initial (E_{init.}), transient (E_{Trans.}) and peak tangent (E_{Peak}) moduli. Both orientation and strain range were statistically significant for tangent E. (G, H) Ultimate tensile strength (UTS) and strain-to-failure (ε_f) for the (G) AV and (H) PV leaflets. The UTS in the RAD direction were significantly less than those in the CIRC direction while the leaflets were more deformable in the RAD direction. Therefore, ε_f values for were significantly greater in the RAD direction versus the CIRC direction.

After mechanical assessment of unseeded scaffolds, we cultured our scaffolds with sheep MSCs for up to 4 weeks. The mechanical properties of the cell seeded tri-layered scaffolds were tested after 2 and 4 weeks. A comparison between E, Yσ and Yε of the cell seeded and unseeded tri-layered scaffolds (Fig.58 A-C) determined that MSCs seeded scaffolds, following 4 weeks cultivation, either retained or
exceeded the tensile modulus (E) and \(Y_\sigma\) obtained at the initial time of culture (In PD direction; 
\[E_{\text{init}}=3.01\pm0.58\ \text{Vs. } E_{4\text{wk}}=2.8\pm0.32 \ \text{MPa and } Y_\sigma_{\text{init}}=0.633\pm0.15 \ \text{Vs. } Y_\sigma_{4\text{wk}}=0.63\pm0.09 \ \text{Mpa}.\] 
The unseeded control scaffolds demonstrated \sim 20\% (2wk) and \sim 35\% (4wk) loss in E and \sim 5\% (2wk and 4wk) decrease in \(Y_\sigma\) values with respect to initial properties. This appeared to be compensated by cell seeding and progressive accretion of ECM proteins on the seeded constructs. This is in line with the positive effect of collagen secretion on the scaffold stiffness which has been reported previously.[46, 51] 
The mechanical properties of tri-layered scaffolds were compared to the mechanical characteristics of native tissues following 4 weeks cultivation (Fig.58 A-C). The initial stiffness of the material matched the transient stiffness of the native tissues, particularly for PV leaflets. This region was chosen based on the strain deformation and stress amplitude that native tissues experience (\(E_{\text{scaffolds/DP}}=2.83\pm0.32\)Vs. \(E_{\text{AV/Circ.}}=3.84\pm0.06\) and \(E_{\text{PV/Circ.}}=2.55\pm0.34 \ \text{MPa}\)). However, the scaffold’s yield stress was lower than native tissue UTS. Native AV and PV tissues experience less stress/tension during the opening and closing cycle and stress distribution on the tissue would never reach to UTS level according to the blood flow pressure described in the equation (1) (calculation for pulmonary arteries (PA)): 

\[(6-1) \quad ((P \times r= 2 \times t \times T))\]

Where \(P\) is the diastolic pressure of 20 mmHg, \(r\) is the PA diameter of 25 mm and \(t\) is the leaflet thickness of 300 \(\mu\)m [46]. Using this equation, the stress amplitude on the leaflet (tension, \(T\)) is \sim 65 kPA which is remarkably lower than the scaffold tolerance, \(Y_\sigma\) (0.633\pm0.094 MPa). During the normal circulation, the leaflets stretch to about 30\% strain during opening and closing. Thus, they are exposed to relatively low stress value in accordance with the stress-strain trend of native leaflets, measured in equation above (on the order of 0.1MPa for PV and 0.25MPa for AV).
Figure 58: The tri-layered seeded & unseeded scaffold mechanical properties, which were assessed in both PD and XD directions initially and during culture and compared with the native tissue stiffness (A) $E_{\text{trans}}$, (B) UTS and (C) strain to failure. Dashed line bars represent unseeded control scaffolds which were incubated in culture medium alone (i.e. $E_{\text{init}}$, $Y_{\sigma}$ and $Y_{\varepsilon}$, and for PD and XD-oriented).

4.1.3. Cell Proliferation and Tissue Formation on Single Layer Microfabricated scaffold, Single Layer Fibrous PGS/PCL Scaffolds and Composite Scaffolds Vs. Native Tissue Composition

To demonstrate the potential of our construct to support ECM formation, biochemical assays were performed following 1, 2, 3 and 4 weeks of MSC cultivation to measure DNA, collagen and GAG content in the scaffolds (Fig. 59 A-C). We compared ECM protein deposition and DNA accumulation of the fibrous PGS/PCL and microfabricated PGS scaffolds as well as the tri-layered composites with the values obtained from native AV and PV. The extracted collagen, GAG and DNA from AV and PV did not differ considerably. However, mean collagen/DNA values (Fig. 60 A-B) were slightly higher in AV compared to PV which could be correlated to the higher stiffness of AV leaflets (Fig. 57) and due to the higher blood pressure in the left ventricular outflow tract in which the AV resides [46].
Figure 59: *In vitro* assessment of ECM generation. (A) DNA content and protein deposition, (B) collagen and (C) GAG production for AV and PV as well as cell-seeded scaffolds (fibrous, microfabricated PGS scaffold and tri-layered composites) cultivated for 4 weeks *in vitro*. DNA content increased with time for all three scaffolds. Collagen and GAG increased significantly with time for tri-layered scaffolds while the collagen and GAG concentrations were relatively constant for fibrous and microfabricated scaffolds. The ECM content and DNA concentration for cell-seeded tri-layered scaffolds, were similar to native tissue, following 4 weeks of cultivation.
DNA content increased on scaffolds during the 4 weeks of culture, confirming MSC proliferation. The highest DNA content was found in the microfabricated scaffolds with large diamond-shaped pores after 4 weeks of cultivation. Interestingly, extracted DNA from tri-layered scaffolds matched the DNA content obtained from frozen native tissues (55.04±9.06 vs. 58.15±6.88 in AV and 50.72±10.44 in PV) (Fig.59 A). These results suggest that the combination of fibers and micropores in tri-layered scaffolds may provide an appropriate niche for cell growth.

The collagen/DNA and GAG/DNA were also compared with the native tissues (Fig.60) and results depicted that following 4 weeks cultivation collagen/DNA values are similar to PV results and a bit lower from AV values. Moreover, GAG/DNA obtained from tri-layered composite after 4 weeks culture are slightly higher than native PV and AV.

To promote collagen synthesis, 82 µg/ml of L-ascorbic acid-2-phosphate was added to the media that was used to culture the cell-seeded scaffolds and collagen and GAG contents were quantified after 2 and 4 weeks of culture. Given the constant values of collagen and GAG contents within the single layer fibrous scaffolds from week 2 to week 4, we concluded that fibers alone did not support adequate tissue.
formation. ECM extraction from tri-layered scaffolds indicated that collagen and GAG content increased considerably with time (P<0.001 for collagen production reported in each time point, and P<0.05 for GAG production between time zero and week 2and 4) (Fig. 59 B-C). GAG and collagen content extracted from tri-layered scaffolds at week 4, as opposed to single fibrous and microfabricated scaffolds, are similar to those obtained from native tissue ECM extraction which can be due to the microstructure of the fabricated composite. Studies have demonstrated that scaffold structure and porosity affect cell-cell and cell-matrix interactions; higher porosity and pore interconnectivity provide adequate surface area for cell growth and ECM generation [75]. A relatively lower amount of DNA and higher amount of collagen and GAG extracted from the tri-layered scaffolds resulted in a higher collagen/DNA and GAG/DNA values for those composite (Fig.60 A-B). This confirmed that the architecture affects the cellular functionality in terms of ECM deposition and tissue formation on 3D constructs [43, 46, 75, 84]. This may indicate that cell signaling in tri-layered scaffolds switched from more proliferation to more ECM secretion. The underlying cause might have been the structural arrangement of the tri-layered scaffold, which resembles native heart valves. Collagen content extracted from tri-layered scaffolds (1153.90±225.93 µg/g wet weight) was considerably higher compared to our previous reported data on double layer PGS microfabricated scaffolds seeded with VICs (i.e. 681.1 ± 63 and 615.1 ± 56 µg/g wet weight, for AVICs and PVICs respectively) [45]. Single layer microfabricated scaffold collagen content was similar to previous data obtained for laser microfabricated PGS scaffolds (736 ± 193 µg/g wet weight) seeded with rat fibroblasts [45] or reported values by Engelmayer et. al in which smooth muscle cells were seeded on scaffolds (i.e., 546 ± 111 µg/g wet weight for the statically incubated samples and 893 ± 133 µg/g wet weight for the cyclically flexed samples).

6.3.5 MSCs VS VICs Seeding

Tri-layered scaffolds were cultured with both sheep MSCs and VICs separately to assess the tissue formation and changes in the mechanical properties following 2 and 4 weeks cultivation Fig.61. No
considerable differences were observed between the mechanical properties of MSC versus VIC seeded scaffolds as shown in Fig. 61 A.

A comparison between DNA content and ECM protein deposition on tri-layered scaffolds seeded with sheep MSCs and sheep VICS did not demonstrate considerable differences except for an increase in GAG content (obtained after 4wk culture) from VIC seeded scaffolds (Fig.61 B-C).

Figure 61: Mechanical, biochemical, and functional properties of MSC- and VIC- seeded scaffolds. (A) Mechanical characteristics of sheep MSC- and VIC-seeded tri-layered scaffolds following 2 and 4 weeks culture, indicating no significant differences between the samples. (B-C) Analogous results were obtained for ECM and DNA content extracted from scaffolds seeded with VICS and MSCs but higher amount of GAG was extracted from scaffolds seeded with VICS after 4 weeks.

6.3.6 Histology and ESEM

Cell and ECM presence was confirmed within the pores of each scaffold by ESEM and H&E staining (Fig.62 A-G). To ensure cell migration into the middle layer of the tri-layered scaffolds and tissue formation inside that layer ESEM images was performed on cross sections of the tri-layered scaffolds (Fig.62 C-D). It was observed that diamond pores were mostly filled with cells and ECM proteins. &E staining of all three layers also confirmed the presence of cells and tissue formation in all three layers of the scaffolds. Of note, Differences between the native tissue sections and cultivated engineered tissues, with respect to the presence of cells and ECM, could be related to the delamination of the layers with loss of cells and tissues during the cryosectioning process (Fig.62 E-G).
Figure 62: ESEM micrograph of cell-seeded scaffolds and histology. (A) Cells and ECM were formed inside the diamond pores of microfabricated scaffolds (B) and on the surface of fibrous scaffolds as observed in ESEM images (C-D) ESEM images of cross-section of cell-seeded tri-layered composite also indicated the formation of a cell layer inside the diamond pores (red scale bars: 100 μm). (E-G) H&E staining of a native aortic leaflet and (E) a tri-layered scaffolds after 4 weeks of cultivation (F) demonstrated the presence of cells ECM. Each layer of the scaffold, individual layers containing cells are shown in (G).
6.3.7 Immunostaining and Thromobogenicity Staining

VICs are quiescent *in vivo* unless activated to proliferate and remodel into myofibroblast-like cells, hallmarked by alpha smooth muscle actin (α-SMA) expression [23]. and expression of matrix remodeling enzymes (Matrix Metalloproteases (MMP’s), Tissue Inhibitor of Metalloproteinase (TIMP’s), and Cathepsins) [285]. The remodeling potential of cells seeded onto our scaffold *in vitro* was observed by positive staining of α-SMA, indicative of a myofibroblast-like differentiation (Fig.63 A). Cell morphology, alignment and orientation were assessed within the scaffolds after 4 weeks of culture by immunostaining for F-actin and DAPI. Tri-layered scaffold architecture arranged the MSCs cells in a preferred direction (toward the fiber direction on the surfaces and the long axis of diamond pores in the middle layer) (Fig.63 B-C). The cells were completely aligned toward the direction of aligned fiber and the long distance of the diamond pores in the middle layers [46]. Considering the collagen and elastin fiber formation in the direction of aligned cell, we can conclude that scaffolds anisotropy was preserved (as reported in the cell seeded scaffolds mechanical properties as well) following the degradation of the construct.

One of the critical challenges in using synthetic or natural based scaffolds for TEHV is the risk of thrombus formation on the scaffolds, which limits the use of bare scaffolds for implantation. To overcome this problem, previous studies were attempted to endothelialize the surface of the scaffolds prior to implantation [44, 48]. Here we conducted thrombogenicity assays as described protocol in [293] on our tri-layered scaffolds. Only a minimal number of platelets (red dots) attached on the surfaces to the scaffolds, which confirmed that the fabricated scaffolds is relatively non-thromogenic (in *in vitro* study) (Fig.63 D).
Figure 63: **Immunostaining, thrombogenicity.** (A) αSMA (green) and DAPI (blue) expression presented on both sides of tri-layered scaffolds. Representative images of MSCs immunostained for actin filaments (green) and nuclei (blue) on the (B) surfaces and (C) middle layers demonstrated the scaffolds structure guided the cell alignment toward the preferred direction on the fibrous side and middle layer. (D) Thrombogenicity assay on the tri-layered composite scaffolds, demonstrating minimal platelet aggregation (red dots) on the surface of the scaffolds.

### 6.3.8 Scaffolds Functionality as a Single Layer Leaflet

To evaluate the tri-layered scaffold’s ability to open and close at the site of action, we designed a simple *ex vivo* experiment to test the potential of these scaffolds as a single leaflet replacement for PV. Water reservoirs were set in a defined position and ventricles and atrium were connected to those to perform the test ([Fig.64 A](fig:64a)). The pulmonary artery was connected to a line, which provided 10 mmHg of pressure during diastole. The flow and cycles were applied by using clamps attached to the tubes. The repetitive cycles of systole and diastole were manually controlled by the clamps with the implant visible during each cycle in real time ([Fig.64 B-C](fig:64bc)). Both single layered fibrous scaffolds and tri-layered scaffolds were examined as PV single leaflet implants. Tri-layered scaffolds had adequate diastolic leaflet coaptation, whereas single layered fibrous scaffolds coapted incompletely during diastole. This could be explained by considering the lower bending stiffness of the thin fibrous structure. Of note, the tri-layered scaffolds were sutured through the top and bottom fibrous layers to the PA to overcome the bending stiffness of tri-layered scaffolds inhibited the scaffolds natural movement during the test. This is in
accordance with the native structure where the fibrosa and ventricularis layers are holding the scaffolds to the sinus wall.

Figure 64: Scaffold functionality as single leaflet replacement. (A) Experimental setup shows the hemodynamic functionality of the scaffolds as single leaflet substitutes in the PV position, assessed in an isolated heart model. (B) Endoscopic video images of the valve closing and opening demonstrated that fibrous scaffolds were insufficient in systole as well as in diastole. (C) Tri-layered scaffolds however, had adequate systolic performance and complete diastolic coaptation.

6.4 CONCLUSION

In an attempt to engineer functional TEHV constructs, a reasonable initial target is to match the native tissue properties, such as cellular and extracellular matrix composition and organization, mechanical stiffness and anisotropy. In this study we attempted to develop a more biomimetic tissue engineered construct, while simultaneously addressing some of the limitations of our previous work. In particular, we first used a novel PGS micro-molding approach that, unlike most micro-molding approaches, did not require the use of clean room facilities. Combining this technique with directional electrospinning, we designed a novel tri-layered scaffold, comprised of diamond-shaped pores and aligned PGS/PCL fibers on the surfaces to emulate the anisotropy and mechanical properties of native leaflets. Furthermore, we isolated primary sheep MSCs and VICs to seed the fabricated constructs. Scaffolds and cell-seeded constructs were mechanically tested and were further characterized for DNA, collagen and GAG content. To the best of the authors’ knowledge, this is the first report on fabricating a TEHV construct that structurally and mechanically matched native tissue leaflets while providing
appropriate support and environmental cues for ECM deposition and cell proliferation. This scaffold is potentially a more suitable replacement than other materials to address the clinical need of tissue engineered heart valves.
7 DESIGN AND TESTING OF A CYCLIC STRETCH AND FLEXURE BIOREACTOR FOR EVALUATING ENGINEERED HEART VALVE TISSUE

Cyclic flexure and stretch are essential to the proper function of native semilunar heart valves and have demonstrated promise in conditioning tissue engineered heart valves. In the current study, a cyclic stretch and flexure bioreactor was developed and tested in the context of the bioresorbable elastomer PGS and biomimetic engineered heart valve tissues comprised of micromolded PGS scaffolds seeded with porcine aortic VICs. Results demonstrated significant effects of cyclic stretch on PGS mechanical properties, including significant decreases in effective stiffness versus controls. In VIC-seeded scaffolds, cyclic stretch elicited significant increases in DNA and collagen that paralleled maintenance of effective stiffness. This work provides a basis for investigating the roles of mechanical loading in the formation of tissue engineered heart valves based on elastomeric scaffolds.

The aim here was to test that our novel stretch/flexure bioreactor can achieve physiological strain rates. The associated experimental findings contribute to the development of a quantitative basis for designing functional tissue engineered heart valves. The rationales for this hypothesis make design and use of a novel cyclic stretch bioreactor in development of engineered cardiac valve tissue.

7.1 DESIGN, STRUCTURE AND MATERIALS OF THE STRETCH/FLEX BIOREACTOR

The bioreactor was based on previous designs [51, 235, 294, 295]. It is actuated by an environmentally-sealed NEMA 17 stepper motor (UltraMotion, Cutchogue, NY), ansi2035 controller (Applied Motion Products, Watsonville, CA), and a custom LabVIEW program (National Instruments, Austin, TX). The motor is coupled to a drive shaft protruding down from the motor through a polycarbonate lid into the center of a cruciform-shaped polycarbonate bath (Fig. 65). The lead has been designed with angle to prevent contamination (Fig. 65 C). The bioreactor size is appropriate enough to fit in any commercially available incubator (Fig. 65 D). Inside the bath, the drive shaft is coupled to an anylon spur gear (7/8" pitch diameter; McMaster-Carr, Santa Fe Springs, CA) and terminates in a polybutylene terephthalate and glass ball bearing (3/4" outer diameter; KMS Bearings, Inc., Anaheim,
This central gear is meshed with 4 identical gears, each of which further meshed with an additional gear emanating along the arms of the bath. From each of these 8 gears protrudes a 1/16" diameter, 316 stainless steel "grip pin" (McMaster-Carr) (Fig.65 E). Each of these 4 pairs of gears and associated grip pins hold a single scaffold or tissue specimen. Excluding the motor, the bioreactor was autoclave sterilized prior to use. Turning the drive shaft clockwise (Fig.65 F) or counterclockwise (Fig.65 G) enabled tensile stretch or flexural deformation of polymer-based samples, respectively.

Figure 65: Schematic of bioreactor design and structure with scaffolds positioning. (A,B) The cruciform-shaped bath houses 8 pairs of gears engaged by a central gear coupled to a stepper motor (3D and top-view). A pin (white arrow) protrudes from the surface of each gear to grip a sample (C) A side view of the bioreactor showing the sliding lid design which helps to prevent contamination (circled). (D) A photograph of the bioreactor setup, including the controller derive. The size is appropriate enough that the bioreactor can be fit in an incubator easily. (E) 3D rendering of a gear pair with a sample suspended between the gears on the grip pins. The ends of PGS samples were sandwiched between looped segments of rubber band (white arrow) and secured with Teflon tape (black arrow) to provide hoops for the grip
pins. (F-G) PGS stretching to 10% strain (top-view, E) and bending to a radius-of-curvature of ~9.1 mm. Gear motions are indicated by black arrows. Scale bars = 1 cm.

In earlier designs, it was found that too many pairs of gears in a row would be problematic, and would result in little or no movement in gears further from the drive motor. Because of this, the design was revised to use a cross-shaped bath, so that it still allowed for 4 samples at a time in each bioreactor, but each pair of gears holding a sample was in direct contact with the central drive gear, ensuring that all samples were stretched for the same amount and time (Fig.65 B) as opposed to previous designs that samples were run in a row far from the deriver shaft (Fig66).

Figure 66: (A-B) Primary design of bioreactor with gears placed in one row while the shaft was driving in the middle.

The design improved overtime based on pilot experiments, to eliminate the friction between the adjacent gears set. Of particular note, the 8 gears situated adjacent the drive shaft gear include 316 stainless steel pins (1/16” diameter) located ~1 mm inside of the gear pitch radius and protruding toward the top of the bath perpendicular to the plane of the gear face (hereby referred to as “grip pins”). Each pair of these gears (4 pairs in total) and associated grip pins are associated with a single specimen of PGS scaffold (thus, the bioreactor can be loaded with a total of 4scaffold specimens at once). Prior to autoclaving, rectangular scaffolds (~25 x 5 x 0.25 mm) will be hold between rubber bands via Teflon tape on either end (Fig.65 E). Scaffolds will be aseptically loaded into the bioreactor using forceps by positioning the holes in the rubber bands one at a time, over the grip pins. Following loading a scaffold
specimen between its respective grip pins, the scaffold can be stretched (up to a maximum of \(~75\%\) strain) by programming a desired rotation of the stepper motor, and thereby rotating the opposing gears of each gear pair in toward the center of the scaffold specimen (of note, for use in related heart valve tissue engineering research, scaffold specimens can also be flexed by rotating the gears away from the scaffold specimen). The typical working volume of the bath is 230 ml of media and the entire device (excluding the stepper motor, which is removable and sterilized by ethylene oxide gas exposure or soaking in ethanol and UV exposure overnight.

7.2 LABVIEW SOFTWARE PROGRAMING

The bioreactor is actuated by an incubator-compatible, environmentally-sealed NEMA 17 stepper motor (UltraMotion, Mattitick, NY) controlled by a digital indexer/controller/power supply (si2035; Applied Motion Products, Watsonville, CA) and a custom virtual instrument and associated graphical user interface developed using LabVIEW software (ver. 9, National Instruments) (Fig.67). The program has been designed to enable the user for controlling the rotation degree of the gears toward the middle shaft, away from middle shaft or a combination of both to apply stretch, flexure or both on the scaffolds. The rotation rate can also be determined through the program. In our design we have two sets of movement rate, any values over 1 lead to the high strain rate according to physiological condition (about 1cycle/sec. depending on other variables set up). There are two more variables that can be controlled during the gear movement; hold at stretch and hold at rest. These two options set the amount of time that the user define to keep the samples at stretch (or flexure) and rest (or flexure) positions, respectively. The overall period of the cycle is determined by considering these two times as well as gear strain rate.
Figure 67: LabVIEW software allows us defining the strain rate, angle of shaft rotation, time the samples can sit at stretched and at rest.

7.2.1 Bioreactor Stretch and Flexure Measurements Programing and Validation

Using LabView, a program was written to run the motor on the bioreactor. For extension, the program specified that the motor would turn the gears in the system with any specific angle of rotation, $\alpha$, which is then correlates to strain values $\varepsilon(\alpha)$, as shown in the derivation below, corresponding to (Fig. 68).
(7-1) \quad d(\alpha) = a \sin(\alpha)

(7-2) \quad \varepsilon = \frac{L - L_0}{L_0}

(7-3) \quad (\alpha) = L_0 + 2 \cdot d(\alpha) = 2 \cdot a \cdot \sin(\alpha)

(7-4) \quad \varepsilon(\alpha) = \frac{L_0 + 2 \cdot a \cdot \sin(\alpha) - L_0}{L_0} = \frac{2 \cdot a \cdot \sin(\alpha)}{L_0}

Where \( d(\alpha) \) is half of the deformation of scaffolds following stretch due to \( \alpha \) degree rotation. \( L_0 \) is the initial length of the scaffold and \( L \) is the length of scaffolds following stretch. And \( a \) is the distance from the center of the gear to the grip pin (~8 mm). This rotation takes place over 1.25 seconds, after which the system holds the sample at extension for ~0.25 seconds, and then returns to the relaxed state over an additional 1.25 seconds.

For the flexing, samples are partially flexed by turning the motor in the reverse direction. The program (timing and movement) can also be set for the flexure condition. Of note, a sample can be stretched and bended simultaneously by arranging the angle of rotation. However, it is also possible to run 2 sets of bioreactors with one programmer derive to apply stretch and flexure stimulation separately. In this case, for flexure stimulation, the gears are oriented such that the attachment pins are on the other side (across the arm of the cross) so that the turning of the gears brings the samples closer together instead of further apart. This allows us to run both extension and flexion samples in the same time in different bioreactors depending on the orientation of each gear pair. The radius of curvature for flexed samples is calculated here, also corresponding to Fig. 68.

(7-5) \quad C = L(\alpha) = L_0 - 2 \cdot d(\alpha) = L_0 - 2 \cdot a \cdot \sin(\alpha)

(7-6) \quad \sin \theta = \frac{c/2}{R_C} = \frac{L_0 - a \cdot \sin \alpha}{r}

(7-7) \quad \frac{\theta}{360} = \frac{\text{arc length}}{\text{circumference}} = \frac{S}{2\pi r}

(7-8) \quad \theta = \frac{90L_0}{\pi R_C}
Figure 68: **Stretch and flexure schematic.** The samples stretched following clockwise rotation of middle shaft and flexed while the shaft moves counter-clockwise. 2d is the deformation of the samples during stretch and flexure. α is the angle which gears rotate. Strain at stretch and radius of curvature at flexure can be defined as a function of α.

To verify the precision of the bioreactor functionality, a PGS cured polymer membrane (at 160°C, 8hr which is elastic and deformable) was positioned on one set of gears inside the bioreactor. Two lines on the polymer were marked with blue marker. The samples were stretched during a trial run and the procedure was recorded with video imaging. The distances between the lines were measured based on pixel sizes by image processing following the procedure and it was found that by 10 degree rotation of the gears, the polymer (with initial 20 mm length) was stretched with 10% strain. The results were consistent for both strain high and low deformation rate (**Fig. 69**).
Figure 69: **Functionality Test**: a PGS membrane cured at (8h, 160°C) was positioned inside the bioreactor and 10° was set for gear rotation in the LabView software. Image analysis demonstrated that 10 degree rotation of the gears is related accurately to the 10% strain of the samples as obtained in the mathematical equations for relation between α and strain.

### 7.3 THE EFFECTS OF CYCLIC FLEXURE ON TEHV SCAFFOLDS

At the most basic level, most tissue engineering involves growing cells on a natural or synthetic biocompatible, degradable scaffold which will then be implanted to replace the problematic native tissue. However, it has been shown that some conditioning of the construct, specific to the tissue in mind, can improve the cellular development and growth of the tissue-engineered construct. The conditioning must be performed in a way to simulate the natural environment of the tissue being engineered.

#### 7.3.1 Introduction

Cyclic flexure and stretch are natural by second routine of semilunar heart valve leaflets; they undergo changes in curvature of up to 0.6 mm\(^{-1}\)\cite{296-298} and tensile strains of 15%(circumferential
direction) to as high as 80% (radial direction) [299, 300] in response to blood flow during opening and transvalvular pressure upon closing. At the organ level, bending and stretching of the leaflets are essential to valve function, minimizing transvalvular pressure gradient during ventricular ejection [301] and maximizing leaflet coaptation and competent valve closure [300]. In heart valve tissue engineering, the independent roles of cyclic flexure and stretch have been under investigation for over a decade [294, 302].

First reported by [187, 188], tissue engineered heart valves (TEHVs) seek to recapitulate native-like form and function through physiologically-motivated combinations of cells, scaffolds, and biophysical stimulation. [189, 303] first demonstrated bioreactor-mediated promotion of TEHV formation. Compared with static culture, pulse duplicator conditioning yielded increases in collagen and DNA content of 1075% and 261%, respectively. Toward dissecting this pressure-flow stimulus into individual constituents, bioreactors were developed for applying flow [119], stretch [302], flexure [51, 294], and combinations thereof [235, 295] to rectangular strips of TEHV based on bioresorbable nonwoven polyester scaffolds [304].

Toward biomimetic TEHVs with anisotropic, elastomeric mechanical properties, laser microablation [45, 226] and micromolding [46] have recently been utilized to fabricate scaffolds comprised of geometrically well-defined pores and elastomeric PGS structural elements amenable to cyclic stretch. We previously demonstrated that micromolded PGS scaffolds are capable of supporting valvular interstitial cell (VIC) attachment and tissue formation in static 3-dimensional (3-D) culture [46]. Here we developed a bioreactor to investigate the independent effects of cyclic stretch and flexure on VIC-mediated tissue formation on micromolded PGS scaffolds.

7.3.2 Effect of Stretch on PGS Membrane Degradation

Toward understanding the effects of cyclic stretch on PGS mechanical properties, the bioreactor was first tested using solid PGS membranes (n=4 per PGS curing condition (8 h at 160°C and 48 h at 120°C), incubation condition (static and cyclic stretch), and incubation time (initial, 1 and 2 weeks)).
PGS membranes cured 8 h at 160°C were tested based on their use in [226]. In that study, PGS scaffolds with accordion-like honeycomb pores and mechanical properties resembling those of myocardium were microfabricated from PGS cured ~8 h at 160°C and, in one experimental group, cyclically stretched (10% strain, 1 Hz) for 1 week at room temperature in phosphate buffered saline (PBS).

In addition, PGS membranes cured 48 h at 120°C were tested, as these curing conditions were demonstrated in the seminal work of [224] to yield a highly compliant, highly elastomeric PGS (i.e., Young’s modulus = 0.28 ± 0.03MPa, strain-to-failure = 267 ± 59 %). Prior to autoclave sterilization, the ends of PGS membranes were sandwiched between looped segments of rubber band and secured by wrapping with Teflon tape to provide a hoop through which the grip pin could be inserted (Fig. 65E).

Approximately 10% strain (ε) was applied to gripped PGS membranes by programming the stepper motor shaft to turn an angle (α)of 8 degrees (0.1396 radians) per Eq. (1), in which r is the radius from the center of the gear to the grip pin (~8 mm) and L₀ is the initial length of the PGS membrane (~22 mm):

\[ \epsilon(\alpha) = \frac{2}{L_0} \alpha \sin(\alpha) \]

Strain was applied via an approximately triangular waveform at a frequency of ~0.36 Hz (1.25 s gear rotation, 0.25 s hold, and 1.25 s gear return).

7.3.3 Cyclic Stretch and Flexure Test on Scaffolds

7.3.3.1 Scaffold Fabrication and Cell seeding

PGS pre-polymer was synthesized by 1:1 molar ratio polycondensation of glycerol and sebacic acid as described previously. Solid PGS membranes ~300 µm thick were cast on sucrose-coated glass slides. Porous, 3-D PGS scaffolds ~300 µm thick and comprised of diamond-shaped pores with long x short axis dimensions of ~260 x 150 µm and ~80 µm-wide struts were fabricated by micromolding.
techniques (Fig. 70 A) as described in Chapter 4. Scaffolds used in this section are the double layered microfabricated PGS scaffolds used in Chapter 3 and cell seeded with porcine aortic VICs (Fig. 70 B).

![ESEM images of scaffolds](image)

Figure 70: **ESEM images of scaffolds**, (A) unseeded double-layer scaffold obtained prior cell seeding and, and (B) seeded double-layer scaffold obtained following cell seeding in the bioreactor.

Porcine aortic VICs were isolated by enzymatic digestion [220] as adapted previously [46]. VICs were expanded in E199 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic (Invitrogen) and utilized between passages 6 and 7. Autoclave-sterilized, micromolded PGS scaffolds (~22 x 5 x 0.3 mm) were soaked for 2 weeks in the above culture medium to help enhance cell attachment upon seeding [46]. The static seeding method utilized here was distinct from the dynamic, rotisserie-based method of our previous studies [45, 46]. Specifically, ~1×10⁶ trypsinized VICs were resuspended in 500 µL of complete medium. In a Costar ultra-low attachment 6-well plate (Corning Incorporated Life Sciences, Tewksbury, MA), 250 µL of this concentrated cell suspension was distributed by pipette onto each side of the PGS scaffold and allowed to attach for 6 h prior to filling the well with 6 mL of medium supplemented with 82 µg/mL of L-ascorbic acid-2-phosphate sesquimagnesium salt hydrate (Sigma) to promote collagen synthesis.
7.3.3.2 Cell Culture in Static and Bioreactor Condition

PGS scaffolds fabricated and seeded with porcine aortic VICs as described in Chapter 4 were cultivated statically for 2 weeks in Costar ultra-low attachment 6-well plates (Corning) prior to either continued static culture in 6-well plates or loading into the autoclave-sterilized bioreactor (n=4 per incubation condition (static, cyclic flexure, and cyclic stretch). Cyclic stretch was applied as described for solid PGS membranes.

Prior to initiating cyclic flexure, VIC-seeded PGS scaffolds were pre-flexed to ensure they bent in only one direction. The stepper motor shaft was programmed to turn an angle $\alpha$ of 10 degrees, providing an initial pin-to-pin distance of $\sim$19.2 mm. Per Eq. (7-11), this yielded an initial radius-of-curvature ($R_c$) of $\sim$12.4 mm,

$$C \theta/s = \sin(\theta), \quad R_c = S 180/\pi$$

where $S$ is the arc length of the VIC-seeded PGS scaffold (i.e., 22 mm), $C$ is the pin-to-pin distance (i.e., the chord length of the approximately circular arc formed by the scaffold bent between the two grip pins), and $\theta$ is the central angle of the circular arc. Eq. (7-11) was solved for $\theta$ using Microsoft Excel Solver and used to calculate the $R_c$. For cyclic flexure, the stepper motor shaft was programmed to turn back-and-forth an additional 8 degrees, yielding a final $R_c$ of $\sim$9.1 mm.

7.3.3.3 Uniaxial tensile mechanical testing

Solid PGS membranes and VIC-seeded scaffolds were tensile tested as described in Chapter 2 using a 5N load cell, and Nexygen™ Plus software (Lloyd Instruments, Ltd., West Sussex, UK). Effective stiffness ($E$) in the 0-10% strain range, ultimate tensile strength (UTS), and strain-to-failure ($\varepsilon_f$) were measured. VIC-seeded PGS scaffolds were tested parallel to the long axis of the diamond-shaped pores.
7.3.3.4 DNA and collagen assays

DNA concentration, [DNA], was quantified using the PicoGreen DNA quantitation kit (Invitrogen) and a Spectramax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) and collagen concentration.

Collagen concentration, [Colagen], was quantified using the Sircol™ collagen assay kit (Biocolor Ltd., United Kingdom) and a Genesys 20 spectrophotometer (Thermo Fisher Scientific).

7.3.3.5 Confocal fluorescence microscopy

VIC-seeded scaffolds were imaged as detailed previously. In brief, formalin-fixed specimens were stained for filamentous (F-) actin using Alexa Fluor 488-phalloidin (Invitrogen), counterstained for cell nuclei by cover-slipping with Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA), and imaged on a Fluoview FV1000 laser scanning confocal microscope (Olympus America, Center Valley, PA), with F-actin and nuclei pseudo-colored green and blue, respectively.

7.3.4 Computational Simulation and FE Analysis

FE analysis was used to simulate the PGS membrane and scaffolds stretched between two gears. To better understand the stress/strain distribution and mechanical stimulation applied on the samples, 3-D model of membrane and PGS scaffolds (with and without cells) was generated in ABAQUS (V6.) (Fig. 71) and cyclic loading was applied on both edges of the model to simulate the cyclic stretch stimulation in the bioreactor. For the membrane, a homogenous polymer with elastic characteristic (Young’s modulus were applied from experimental data for membrane cured for 8h). For solid membrane, 26116 Hexahedral elements (8 nodes) and for scaffolds, 150264 Tetrahedral elements (4 nodes) were used.
Figure 71: FE analysis for PGS membrane and unseeded and seeded single layer PGS scaffolds stretching simulation in the bioreactor to obtain the stress and strain distribution on the samples during a stretch cycle.

7.4 RESULTS

Basic bioreactor operation was tested. Bare and cell-seeded PGS scaffolds were gripped and stretched to modest (i.e. 10%) strain. The custom LabVIEW program was successfully demonstrated to control the speed and scaffold strain.

7.4.1 Bioreactor Operation and Sterility

Following autoclave sterilization, the bioreactor demonstrated maintenance of sterility in 3 experiments with solid PGS membranes and 2 experiments with VIC-seeded scaffolds, each of which conducted in complete culture medium at 37°C for 4 days. The LabVIEW program allowed for reproducible control of tensile and flexural deformations through specification of stepper motor parameters based on kinematic equations relating tensile strain and flexural radius-of-curvature to stepper motor rotation.
7.4.2 Effect of Bioreactor Conditioning and Incubation Time on Solid PGS Membranes

Stiffness (E) of solid PGS membranes cured 8 h at 160°C depended significantly on both bioreactor condition ($p = 0.0223$) and incubation time ($p < 0.0001$) (Fig. 72A). Compared to initial E (1.00 ± 0.11 MPa), E decreased: ~24% to 0.76 ± 0.12 MPa with 1 week static incubation; ~40% to 0.60 ± 0.07 MPa with 1 week cyclic stretch; ~34% to 0.66 ± 0.12 MPa with 2 weeks static incubation; ~50% to 0.50 ± 0.07 MPa with 2 weeks cyclic stretch. UTS depended significantly on incubation time ($p = 0.0001$), but the effect of bioreactor condition ($p = 0.1777$) did not reach statistical significance (Fig. 72B). Compared to initial UTS (0.46 ± 0.06 MPa), UTS decreased: ~23% to 0.36 ± 0.08 MPa with 1 week static incubation and ~45% to 0.25 ± 0.05 MPa with 1 week cyclic stretch. By contrast, UTS values of 2 weeks static incubation (0.44 ± 0.05 MPa) and cyclic stretch (0.43 ± 0.06 MPa) did not appear to be significantly different from initial properties. The $\varepsilon_i$ depended significantly on incubation time ($p < 0.0001$); however, the effect of bioreactor condition ($p = 0.1126$) did not reach statistical significance (Fig. 72C). Compared to the initial $\varepsilon_i$ (0.67 ± 0.19), no differences were apparent at 1 week in either the static (0.75 ± 0.25) or cyclic stretch (0.65 ± 0.22) groups. By contrast, $\varepsilon_i$ increased ~100% to 1.27 ± 0.18 with 2 weeks static incubation and ~170% to 1.82 ± 0.26 with 2 weeks cyclic stretch.

Stiffness (E) of solid PGS membranes cured 48 h at 120°C depended significantly on both bioreactor condition ($p = 0.0041$) and incubation time ($p < 0.0001$) (Fig. 72D). Compared to initial E (0.27 ± 0.05 MPa), E decreased: ~21% to 0.21 ± 0.06 MPa with 1 week static incubation; ~67% to 0.09 ± 0.04 MPa with 1 week cyclic stretch; ~47% to 0.14 ± 0.02 MPa with 2 weeks static incubation; ~67% to 0.09 ± 0.03 MPa with 2 weeks cyclic stretch. UTS depended significantly on bioreactor condition ($p = 0.0186$), but the effect of incubation time ($p = 0.2403$) did not reach statistical significance (Fig. 72E). Compared to initial UTS (0.21 ± 0.03 MPa), UTS did not appear to change with 1 week static incubation (0.23 ± 0.06 MPa), but decreased ~40% to 0.13 ± 0.03 MPa with 1 week cyclic stretch. Similar to 1 week static incubation, UTS values of 2 weeks static incubation (0.22 ± 0.06 MPa) and cyclic stretch (0.19 ± 0.03 MPa) did not appear to be significantly different from initial properties. The $\varepsilon_i$ depended significantly
on incubation time ($p < 0.0001$); however, the effect of bioreactor condition ($p = 0.1162$) did not reach statistical significance (Fig. 72C). Compared to the initial $\varepsilon_f (1.60 \pm 0.21)$, $\varepsilon_f$ increased: $\sim38\%$ to $2.21 \pm 0.73\text{MPa}$ with 1 week static incubation; $\sim75\%$ to $2.79 \pm 0.58\text{MPa}$ with 1 week cyclic stretch; $\sim156\%$ to $4.09 \pm 0.83\text{MPa}$ with 2 weeks static incubation; $\sim220\%$ to $5.10 \pm 1.56\text{MPa}$ with 2 weeks cyclic stretch.

Figure 72: Tensile properties of PGS membrane cured 8 h at 160°C (A-C) or 48 h at 120°C (D-F). Error bars = standard error of the mean (SEM); $p < 0.05$ were considered significant.

7.4.3 Static Stretch

Single layer microfabricated PGS scaffolds seeded with aortic VICS and cellular proliferation was visualized with microscope under 10% strain over the period of one week. As observed and expected, the stretch resulted in cellular alignment toward the direction of stimulation. As shown in Fig. 73, cells started to grow and proliferate toward the long axis of diamond, the direction the scaffolds was stretched and fixed at 10% strain.
Figure 73: **Static Stretch**: Single layer PGS microfabricated scaffolds stretched at 10% strain deformation and cellular arrangement was visualized over a period of 7 days culture. As shown, cells started growing around the diamond edges toward the middle of the pores aligned in the direction diamond long axis (direction of applied stretch).

### 7.4.4 The Independent Role of Cyclic Stretch and Flexure on VIC-Seeded PGS Scaffolds

Confocal fluorescence micrographs of cyclically stretched VIC-seeded scaffolds revealed cells oriented within pores, along PGS struts (**Fig. 74 A**), and, in some pores, interleaved between scaffold layers (**Fig. 74B**). Qualitatively, within the set of micrographs evaluated, no clear differences were readily apparent between static and cyclic flexure groups (not shown) and the cyclic stretch group.

[DNA] depended significantly on culture condition (**Fig. 74C**). Compared to static culture (46 ± 24 μg/g wet weight), [DNA] increased ~158% to 118 ± 10 μg/g wet weight with cyclic flexure, and
~191% to 133 ± 35 µg/g wet weight with cyclic stretch. No significant difference was apparent between cyclic flexure and stretch. [Collagen] depended significantly on culture condition (Fig.74D). Compared to static culture (873 ± 311 µg/g wet weight), [collagen] increased ~423% to 4564 ± 1660 µg/g wet weight with cyclic stretch. By contrast, the ~227% increase in [collagen] to 2858 ± 1152 µg/g wet weight in the flex group did not reach statistical significance by Tukey’s post-hoc test. While a trend of increased [collagen]/[DNA] was apparent on going from static to cyclic stretch conditions, with ratios ranging from 19 ± 12 (static), 24 ± 10 (flex), and 34 ± 15 (stretch) (Fig.74E), the effect of culture condition did not reach statistical significance.

With a border line p-value of 0.0522, the effect of culture condition on E was nearly, but not formally, significant (Fig.74F). In particular, compared to the initial E of the scaffold itself (0.76 ± 0.05 MPa), an ~40% decrease in E was apparent with static culture (0.46 ± 0.07 MPa), but did not reach statistical significance by Tukey’s post-hoc test. By contrast, no difference in E was apparent between the initial E of the scaffold itself and that of the cell-seeded scaffold under cyclic flexure (0.73 ± 0.21 MPa) or cyclic stretch (0.65 ± 0.12 MPa) conditions. Mean UTS depended significantly on culture condition (Fig.74G). Compared to the initial UTS of the scaffold itself (0.24 ± 0.03 MPa), UTS dropped an average of ~47% in the static (0.11 ± 0.04 MPa), cyclic flexure (0.12 ± 0.02 MPa), and cyclic stretch (0.14 ± 0.02 MPa) groups, with no difference apparent between these three groups. The initial ε_f of the scaffold itself appeared to decrease upon static culture, but the effect did not reach statistical significance. While a trend of increased ε_f was apparent on going from static to cyclic flexure conditions, with values ranging from 0.27 ± 0.05 (static), 0.49 ± 0.25 (flex), and 0.63 ± 0.31 (stretch) (Fig.74H), the effect of culture condition did not reach statistical significance.
Figure 74: **PGS scaffolds culturing in bioreactor**: confocal micrographs of cyclically stretched, VIC-seeded scaffolds (A,B). Cells oriented within pores, along PGS struts (white arrows, A), and, in some pores, interleaved between scaffold layers (white arrow, B). Green = F-actin; blue = cell nuclei, scaffold struts (PGS autofluorescence). Scale bars = 120 microns. DNA (C) and Collagen (D) concentrations, collagen-to-DNA ratio (E), and tensile properties (F,G,H) of VIC-seeded scaffolds. Error bars = standard error of the mean (SEM); *p* < 0.05 were considered significant (one-way ANOVA). Annotations indicate significant differences between means (Tukey’s post-hoc test): initial (*), static (#), flex ($) .

Confocal images confirmed that cells on the scaffolds cultured in the bioreactor were more aligned toward the direction of stretch/flexure versus the samples that were cultured statically with no mechanical stimulation. **Fig.74** depicts confocal images of F-actin staining for samples cultured in static condition (A-B) and a Z-stag images of F-actin stained scaffolds cultured under stretch condition. Cells are mostly aligned in the diamond pores and particularly on the surface of the scaffolds stretched in the bioreactor while there is no cellular alignment evident on the scaffolds cultured in static condition.
Confocal micrographs of static (A-B) and cyclically stretched, VIC-seeded scaffolds (Z-SET images) (C-E). Cells oriented within pores, along the cyclic stretch direction, while samples cultured at static condition mostly included randomly oriented cells especially on the surface of the scaffolds.

7.4.5 Finite Element Analysis to Determine Stress and Strain Distribution on Scaffolds and PGS Membrane

Using FE analysis, we were able to define the stress and strain distribution over PGS membrane and scaffold’s struts following stretch in the scaffolds (seeded and unseeded scaffolds) for 20% strain. Those data were also obtained in tissue regions, inside the diamond pores, (considering E:14 kPa stiffness for cells). It was found that stress is most concentrated at the connection point of the scaffolds struts which is similar to the findings in Chapter 3. The stress and strain distribution on the PGS membrane has been reported from 4 points of (one in the middle and 3 points on the edge of the scaffolds (Fig. 76). FE analysis confirmed that 20 degree rotation of the gears resulted in 20% strain deformation as shown in graphs. Except for node 3, stress amplitudes were similar for other points and are in the range of 0.06
186 MPa which is lower than polymer tolerance (UTS reported in chapter 2). About 0.6 MPa stress was reported on the membrane surface during the cyclic stretch with low strain rate (~2 HZ).

Figure 76: **FE analysis on PGS membrane.** (A) Sample stretched at 20% strain was simulated with FE computational modeling (B) to obtain the stress and strain distribution on the membrane. Stress and strain contour were similar and showed a uniform stress and strain distribution on a 4mm by 7mm PGS membrane sheet. (C) 4 nodes on the membrane were chosen and the cyclic strain (D) and stress (E) values were measured and depicted in. As expected, 20% strain was obtained on the PGS membrane except for a corner points that due to interaction with boundary force experienced larger strain and stress.

**Fig. 77 A-E** demonstrates the cyclic stress and strain distribution and amplitude obtained on four regions of the scaffolds (inside the pores and on the scaffolds struts) in a period of one stretch cycle. For cell seeded scaffolds, it was observed that majority of stress amplitude is distributed on the scaffolds due to higher stiffness of the scaffolds versus cells elasticity in the pores. Therefore, it is mostly the scaffolds that stand for the imposed stretched. Contour of stress and strain deformation on the cells were also
depicted on three regions of the scaffolds. It should be considered that allocating a homogenous elastic characteristic for material inside the scaffolds diamond pores (as tissue and cells) is one of the limitation in FE analysis that is far from reality.

![Figure 77](image_url)

Figure 77: **FE analysis on cell seeded single layer microfabricated PGS scaffolds**: (A) demonstrates the zoom in strain and stress distribution on the cells inside the diamond pores and (B) stress/strain contour on the scaffolds struts. Strain and stress were measured for 4 nodes on the cells inside the pores and on the scaffolds struts in one cycle (C-F). Due to higher stiffness of the scaffolds, it is the main element for holding the cyclic force during the composite stretch, therefore higher magnitude of stress was obtained on the scaffolds compared to cells inside the diamond pores.

### 7.5 DISCUSSION

TEHV bioreactors have evolved from pulse duplicators to promote the formation of implantable tri-leaflet constructs [189, 303], to *in vitro* tools for studying the independent or coupled effects of cyclic
stretch, flexure, and flow in rectangular TEHV strips [51, 119, 235, 302], to advanced pulsatile flow and/or pressure systems capable not only of promoting the formation of tri-leaflet constructs, but also of more complex functions, including the potential for decoupling flow and pressure stimuli [121, 148], imaging of leaflet deformations [112, 305-308], and measuring of mechanical properties [309]. Depending on their design criteria, bioreactors are associated with specific advantages and limitations.

With a goal of facilitating aseptic sample loading, the current bioreactor used a top-mounted stepper motor in lieu of a side-mounted linear actuator. In contrast to a previous cyclic flexure-stretch-flow (FSF) bioreactor [235, 295], the bottom-mounted, gear-based mechanism for converting rotary to linear motion allowed samples to be positioned onto the grip pins entirely from above. This advantage, however, came at the expense of the added complexity of the gear-based actuation system itself. In a prototype version of the bioreactor (not shown), the gear pairs were arranged in a linear array along the length of a narrow, rectangular bath, in which the stepper motor and drive shaft were positioned asymmetrically at one end of the array. In that prototype, gear slippage associated with axial play intrinsic to the ball bearings limited reproducible function. In the final design, symmetric positioning of the stepper motor drive shaft at the center of the cruciform-shaped bath overcame the limitations of the linear array, but with the limitation of a smaller sample capacity.

Another limitation of the bioreactor was its pin-based gripping mechanism. In a previous FSF bioreactor, [235, 295] introduced a “spiral-bound” method for gripping nonwoven PGA-based scaffolds between pins, in which thin, stainless steel wire coils were threaded onto the ends of rectangular nonwoven scaffold strips, providing hoops through which the grip pins could be inserted. However, due to the elasticity of PGS and potential for stretching and/or tearing at the points of wire insertion, a different method was applied in the present study. Based on the use of rubber band pads to distribute force over the surface of PGS during gripping and stretching [226], rubber band hoops were secured to the ends of PGS membranes and scaffolds by Teflon tape, providing a practical, albeit comparatively inelegant, means of gripping (Fig. 65E). Other approaches, such as rolling the ends of a partially cured
PGS membrane and curing it to itself to form a hoop, or molding sleeves into the PGS capable of accommodating the grip pins, may be pursued.

PGS was introduced by [224] and its biomedical applications have recently been reviewed by [225]. Initial tests of the bioreactor, in which the effects of cyclic stretch on solid PGS membrane mechanical properties were evaluated, offered confirmation of and insights into the results of previous studies. For example, [226] observed a significantly lower E in porous, accordion-like honeycomb PGS scaffolds cyclically stretched (10% strain, 1 Hz) for 1 week at room temperature in PBS (46 ± 4 kPa) compared to control scaffolds soaked statically for 3 weeks (85 ± 7 kPa). In the present study, similarly cured solid PGS membranes (i.e., 8 h at 160°C) exhibited greater decreases in E under cyclic stretch (10% strain, ~0.36 Hz) than static conditions. With the ongoing introduction of evermore promising new PGS-like elastomers [310, 311], mechanically tunable PGS scaffold fabrication techniques [86, 144, 227, 312-314], and computational models capable of predicting PGS scaffold mechanical properties [315, 316], it is clear that evaluation of these materials under physiologically-relevant modes of mechanical loading will be important in TEHVs and other applications.

In addition to cyclic stretch in VIC-seeded PGS scaffolds we also evaluated effects of cyclic flexure. Cyclic flexure has been demonstrated to play an independent role in both nonwoven PGA scaffold degradation [294] and engineered heart valve tissue formation from vascular-derived cells [51] and bone marrow-derived mesenchymal stem cells [235, 295]. In particular, [51] demonstrated that cyclic flexure can stimulate significantly increased (+63%) collagen content, as well as a more homogeneous transmural cell distribution, compared to static controls, and applied structural-based modeling to characterize the flexural mechanics of engineered heart valve tissues based on nonwoven scaffolds [238] and the nonwoven scaffolds themselves [317]. More recently, others have begun to emphasize the design of scaffolds with heart valve leaflet-specific flexural characteristics, including [318], who designed electrospun scaffolds tuned to the flexural properties of native heart valve leaflets, and [319], who tested
laminated poly(ethylene glycol) diacrylate (PEGDA) hydrogels. Underlying the effects of cyclic flexure and stretch are the VICs themselves.

VICs have been implicated in the repair and remodeling of collagen, presumably offsetting mechanical fatigue and mediating leaflet homeostasis. In support of this hypothesis, bioreactor cultivation of porcine aortic valve leaflet tissue has demonstrated that cyclic stretch can elicit an activated, contractile VIC phenotype and increased collagen synthesis [320, 321], with valve-, layer-, and direction-specific extracellular matrix interactions being the prime candidate factors governing VIC deformation and mechano transduction [207, 208, 322, 323]. Cyclic stretch of tissue cultured VICs has provided further insight into their roles in collagen synthesis and alignment [127, 217] and in disease, with strain dependent expression of calcification factors [212, 324].

As eluded to earlier in the context of organ-level valvular function, the implications of cyclic stretch and flexure in TEHVs extend beyond bioreactors. For example, in the repair of congenital heart valve lesions in pediatric patients, the size of the pulmonary outflow tract and leaflets needs to compensate for the somatic growth of the patient [325]. While it is challenging to experimentally decouple the influence of cyclic stretch from other potential mediators of leaflet growth in vivo, [326] directly linked compensatory mitral valve leaflet growth to chronic stretch5 weeks post-infarction in ischemic mitral regurgitation. Thus, longer term in vitro studies of cyclic stretch and flexure on biomimetic TEHVs based on VICs may shed light on mechanisms involved in leaflet growth, by offering the potential to test the effects of factors such as engineered tissue stiffness, anisotropy, and elasticity.
CONCLUSION AND FUTURE DIRECTIONS

8.1 SPECIFIC IMPLICATIONS FOR HEART VALVE TISSUE ENGINEERING AND CONCLUSORY REMARKS

Recognizing that incremental improvements in current replacement valves are unlikely to solve the quality of life and life expectancy problems facing children with congenital valvular lesions, it is believed that an autologous TEHV, due to the potential for growth and self-repair, will be the most viable option for a permanent solution to pediatric valve replacement in the future. Though significant advances were made by applying off-the-shelf scaffolds in constructing TEHV, these collective experience, including the most recent 20 week implantation results in a sheep model [44], suggest that we have reached an impasse. Interestingly, evidence suggests that the primary path in moving toward a fully functional TEHV, and thus the most likely route to a future strategy for the permanent repair of pediatric valvular lesions, depends on the switch to a more biomimetic scaffold. Toward clinical translation, Sutherland et al. [139] demonstrated long-term function of the TEHV, constructed by seeding similar nonwoven textile scaffolds with relatively noninvasively accessible bone marrow-derived MSCs. An ovine heart valve was replaced by a TEHV and showed function similar to that of the native valve and tissue composition after five months (Sodian et al., [327]; Hörstrup et al., [52]). However, the scaffolds could not stand the high pressure in the aortic systemic circulation. Recent advances by Gottlieb et al. [44] refined the original paradigm while simultaneously highlighting some of the principal limitations: the high stiffness, inelasticity, and complex micromechanics of the replaced TEHV [50, 55]. This lead to concern as to whether or not it is required for scaffolds to match native tissue’s mechanical characteristics initially or if a durable, functional TEHV can also be achieved by in vivo remodeling and adaptation. Therefore, for future studies, a most important question will be: how good is “good enough” for initial mechanical and structural characteristics of TEHVs in vivo to survive and replicate function of replaced native valves. As demonstrated in this study, we attempted to develop such a biomimetic scaffold using a computational-design and laser microfabrication, micromolding and electrospinning approaches. The
scaffold materials were a highly biocompatible, mechanically-tunable, bioresorbable elastomer PGS and plastic PCL. In addition, we showed that our finite element models and FE simulations can be used to computationally design microfabricated PGS scaffolds mimicking the anisotropic mechanical properties of native heart valve leaflet tissues. In further achievement, a novel stretch and flexure bioreactor, capable of applying physiological stretch and strain mechanical stimuli, was designed and tissue development was evaluated following scaffold culture in the bioreactor. Thus, in the proposed research, we aimed to push toward a fully functional TEHV by capitalizing on biomimetic, computationally-designed Tri-layered microabricated/microfibrous scaffolds. The stimulus for fabrication of the aforementioned, qualified TEHVs stemmed from requirements for implantation in human patients.

8.2 DESIGN CRITERIA FOR SCAFFOLDS FOR TISSUE ENGINEERED HEART VALVES

1. Mimic biomechanical properties of native valve tissue
   a. Biaxial or uniaxial stress-strain characteristics, including anisotropy (greater stiffness in the circumferential than in the radial direction) and strength and strain in the range of leaflets functionality.
      i. Elasticity
      ii. Anisotropy

   The native tissues’ mechanical properties were measured and the required stiffness, strength and strain that should be matched and held by scaffolds based on those values were reported in Section 6.3.4. We aimed to obtain two important characteristics of native tissue; elasticity and anisotropy. Using PGS and a combination of PGS/PCL as the base of the scaffolds material, along with applying in microfabrication (with computationally designed diamond pores) and directional electrospinning method for creating scaffolds enabled us to achieve both important mechanical properties of native tissue which affect the leaflet functionality.

   b. Flexural stiffness: While we did not quantify the bending stiffness of our scaffolds, we were convinced that our material comprised proper bending stiffness required for
functioning as single heart valve leaflets by testing the scaffolds opening and closing during single leaflet replacement in Section 6.3.9 and in flexure bioreactor testing in Section 7.4.4

2. Cell compatibility and toxicity:
   a. Allow initial cell attachment and proliferation (non-toxic)
   b. Non-toxicity of degradation products
   c. Biocompatibility and stability of the material

Through this study we presented cultivation studies with different cell types. More specifically, we evaluated the capability of skin fibroblast, VICs and bone marrow derided MSCs to promote tissue development on scaffolds for TEHV. Designed microfabricated PGS scaffolds and microfibrous PGS/PCL scaffolds were used individually and in combination (tri-layered structure) to generate scaffolds with appropriate anisotropy and physical characteristics that matched native tissue structure and mechanics.

While the current study emphasized scaffolds based on the bioresorbable elastomer PGS, a variety of other promising scaffold materials-of-construction are currently under development in the context of TEHV [253, 328]. In particular, studies have aimed to create scaffolds mimicking the structural complexity of heart valves while providing an appropriate environment for cellular growth and ECM generation in vitro [40, 42, 44-46, 52]. However, some drawbacks associated with these engineered scaffolds have prevented them from successfully translating to in vivo models. These limitations include unnaturally large stiffness (e.g. non-woven scaffolds) [44, 50, 55], lack of anisotropic characteristics (e.g. homogenous fibrin gels or fibrous scaffolds comprising of random fibers) [41, 42, 47, 58], non-fibrous structures with large pores [43, 45, 46], plastic deformation [47, 57] and the inability of the scaffolds to be sutured (e.g. hydrogel based scaffolds and microfabricated scaffolds) [40, 286].

3. Rate of biodegradation
a. Controlled degradation rate during the tissue remodeling. In an ideal case, the scaffolds degradation rate should be similar to the tissue formation rate in order for scaffolds to maintain its structural and mechanical integrity.

b. Sufficient duration of retention of mechanical integrity and mechanical characteristics to allow/promote ECM production by cells.

   i. Mechanism of biodegradation in cellularized environment should result in non-cytotoxic products
   ii. Degradation should occur with alteration in mechanical behavior that promotes formation of ECM with appropriate mechanical characteristics.

Scaffold anisotropy was largely retained upon cultivation; while the mechanical properties of unseeded scaffolds progressively diminished (mostly through hydrolysis process), the cell-seeded scaffolds either retained or exceeded initial mechanical properties. Retention of mechanical properties in cell-seeded scaffolds paralleled the accretion of collagen, which increased significantly following 4 weeks cultivation.

4. Cellular alignment and tissue architecture

   a. Fiber size < 5-8 microns to promote cell recognition of “growth on a fiber” rather than a flat surface.
   b. Provide or allow incorporation of native ECM adhesion signaling motifs

Native semilunar heart valve leaflets are anatomically complex [4], belying what upon cursory inspection appear to be simple physiologic check valves. Indeed, physiologic mechanical function of native heart valve leaflets compared with current replacement valves derives principally from their specialized collagen fiber network. Nevertheless, a comprehensive micro-anatomical atlas of the native semilunar heart valve leaflet collagen fiber network has yet to be fully realized. Sacks et al. used a small angle light scattering approach [329] to quantify local variations in collagen fiber orientation and applied the technique to engineered tissues [175]. More recently, Doerhing et al. [330] used elliptically polarized light microscopy to image the meso-scale structure of the valve leaflet. Toward promoting native-like
collagen fiber alignment in the context of TEHV based on microfabricated scaffolds, Engelmayr et al. [82] demonstrated a positive correlation between the aspect ratio of microfabricated rectangular pores and collagen fiber alignment in a non-degradable scaffold material and noted advantages of diamond-shaped pores in promoting more reproducible cell and collagen alignment.

In the current study, cytoskeletal stress fiber organization was evident within the VICs and MSCs filling and spanning the diamond-shaped pores of the cell seeded PGS scaffolds and on the surface of microfibrous PGS/PCL scaffolds comprised of aligned fibers, which was demonstrated by F-actin staining of the cultured scaffolds and cell alignment was explicitly quantified (as for microfabricated PGS scaffolds using a Fast Fourier Transform (FFT)-based method (Chapter4) and using image J software (Chapter 5) for PGSPCL fibrous scaffolds). Tri-layered scaffold architecture arranged the MSCs in a preferred direction (toward the fiber direction on the surfaces and the long axis of diamond pores in the middle layer) (Fig.63 B-C). The cells were completely aligned toward the direction of aligned fiber and the long distance of the diamond pores in the middle layers [46]. Considering the collagen and elastin fiber formation in the direction of aligned cell, we can conclude that scaffolds anisotropy was preserved (as reported in the cell seeded scaffolds mechanical properties as well) following the degradation of the construct.

5. Fabrication parameters
   a. Porosity to allow cellular incursion into scaffold (>30 microns)
   b. Capability for fabrication into shape replicating native leaflet shape
   c. Capability for dimensional alteration at time of surgical implantation (ability to be “cut to fit” local anatomic variations)
   d. Suture retention and ultimate tensile strength
   e. Ability to incorporate “reinforcing” fibers into scaffold structure with orientation similar to native valve collagen fiber orientation (i.e., 30° angle from horizontal [331])
Toward designing scaffolds with improved elasticity and structural-mechanical anisotropy similar to native semilunar valve leaflets, Courtney et al. introduced an electrospun poly (ester urethane urea) scaffold exhibiting a biaxial mechanical response similar to native valve leaflet tissue [140]. The processes of fabricating and characterizing these electrospun elastomeric scaffolds were further refined by Amoroso et al. [318], which accounted for the influence of “micro-integrated” particles (e.g., cells), as introduced by Stankus et al. [332]. Electrospinning, however, is an inherently stochastic process in which control of microstructural morphology is possible only in an average sense. Sales et al. [143] first investigated the utility of PGS foam in the context of TEHV. One limitation of PGS foam, however, is its low stiffness (4-8 kPa), when compared with native valve leaflet tissues and effectively random pore structure. Toward rendering PGS scaffolds with geometrically well-defined microstructures and native-like anisotropic mechanical properties appropriate for cardiac muscle applications, Engelmayr et al. [72] recently developed a laser ablation approach to microfabricate PGS scaffolds with an accordion-like honeycomb pore structure capable of matching the anisotropic stiffness of right ventricular myocardium while promoting native-like anisotropic heart cell alignment.

In particular, we first used a novel PGS micro-molding approach that, unlike most micro-molding approaches, did not require the use of clean room facilities. To improve that design toward fabricating a more biomimetic scaffold, a novel scaffold containing fiber structures was fabricated resembling the ECM networks in the native heart valve leaflet. Directional electrospinning was used to create tunable anisotropic composite materials with varying ratios of PGS:PCL. The human VICs were found to be organized and aligned along the anisotropic axes of the composite TEHVs. We demonstrated that scaffold structure affects the cellular arrangement considerably, whereas the substrate stiffness (in the range of 4-9 MPa) has minimal effect on the cell alignment and is supportive across the range tested. VIC attachment, viability and proliferation followed similar trends for each polymer ratio, though there was a considerable difference between these results and those obtained with human and porcine cells. This study was extended to further design a more biomimetic scaffold that eliminated the fibrous scaffold limitations, despite being constructed of the fibrous scaffolds by a combination of structures, (e.g., inability to form a
3D construct due to small porosity of the scaffolds). Thus, we designed a novel tri-layered scaffold with elastomeric and anisotropic properties inspired by the structure and mechanics of native leaflets. A semi-automatic layer-by-layer assembly was applied to fabricate the biocompatible and mechanically tunable 3D construct. We hypothesized that combining PGS/PCL microfibers and microfabricated PGS in a tri-layered construct would provide elastic and anisotropic characteristics for the 3D architecture which would match structural and mechanical properties of native leaflets while simultaneously supporting controlled cellular growth and tissue formation within a guided architecture. Scaffolds and cell-seeded constructs were mechanically tested and were further characterized for DNA, collagen and GAG content. To the best of the authors’ knowledge, this is the first report on fabricating a TEHV construct that structurally and mechanically matched native tissue leaflets while providing appropriate support and environmental cues for ECM deposition and cell proliferation. This scaffold is potentially a more suitable replacement than other materials to address the clinical need of tissue engineered heart valves.

6. Computational modeling to develop TEHV

Mathematical modeling (e.g., computational models [333, 334], analytical models [50, 55]) has been applied in the context of TEHV scaffold design. Prior to the current study, however, a TEHV scaffold had yet to be explicitly fabricated from an analytical or computational model-based design. Jean and Engelmayr [107] developed finite element simulations capable of retrospectively predicting the mechanical properties of the laser microfabricated PGS scaffolds and collaborators Guillemette et al. [144] and Park et al. [84] demonstrated, using an identical system to that used in the current study, the utility of a solid-state LSX-213 laser microablation system in rendering microfabricated PGS scaffolds. Specifically, Guillemette et al. demonstrated advantages of combining micro-molding and laser microablation technologies [144], while Park et al. developed and demonstrated multi-layered laser microablated PGS scaffolds [84]. Of note with respect to alternative microfabrication approaches, Hollister et al. [180] had considerable success applying finite element simulations and PGS micro-molding techniques to cartilage tissue engineering. Collectively, these efforts suggest that combining
computational simulations with PGS microfabrication approaches might be suitable for designing PGS scaffolds for a variety of soft tissues, including recapitulating in TEHV the complex collagenous microanatomy of native heart valve leaflets.

Through this study, we were able to model the structural mechanics of a PGS scaffold with uniform diamond shaped pores. Furthermore, FE analysis was used to develop a correlation between the scaffold’s pore geometry /strut width and the scaffold’s stiffness to determine an appropriate structural design that resembles native leaflet’s stiffness and anisotropy.

7. TEHV development in bioreactor
   a. Controlled mechanical stimulation while mimicking physiological conditioning of native heart valve leaflets.
   b. Simple design for fabrication, relatively cheap and reliable sterility

Considering the fact that physiological function of heart valve leaflets is biomechanical, the potential effects of mechanical factors on TEHV development demand further investigation though the benefit of such preparation condition compared to static incubation has been demonstrated [78, 111, 122]. However, before any THEV design is considered safe for clinical application, it is necessary to evaluate its function in a system mimicking the in vivo mechanical behavior. Heart valve leaflets and their constituent interstitial cells function normally in a mechanically dynamic state of homeostasis [107]; Huang et al. demonstrated that the deformation of aortic valve interstitial cells in situ within leaflet tissues correlate positively, though in a complex, nonlinear fashion, with diastolic transvalvular pressure and associated tissue strains [108]. Hoerstrup et al. [180] was the first to demonstrate the benefit of simulating this native dynamic mechanical environment in the in vitro bioreactor conditioning of TEHV [90,109]. Cyclic mechanical loading, in particular large strains, as demonstrated by Mol et al. [110], has been shown to promote native valve-like collagen fiber orientation in TEHV constructs based on relatively inelastic nonwoven scaffolds [111]. It is thus expected that bioreactor application of cyclic mechanical loading to TEHV materials based on microfabricated, elastomeric PGS scaffolds will likely further
enhance preferential cell and collagen alignment. Also of note, the feasibility of cyclically stretching microfabricated elastomeric PGS scaffolds was demonstrated via \textit{in vitro} fatigue studies in the absence of cells [29]. Importantly, the geometrically well-defined pore shapes of microfabricated PGS scaffolds are expected to facilitate finite element simulations of bioreactor-mediated mechanical loading, such as simulated previously for mesenchymal stem cell-seeded collagen gels by Pfeiler \textit{et al.} [170].

Here, we developed a novel bioreactor capable of cyclically stretching or bending microfabricated elastomeric PGS scaffolds. The aim here was to test the hypothesis that our novel stretch/flexure bioreactor can achieve physiological strain rates. The associated experimental findings contribute to the development of a quantitative basis for designing functional tissue engineered heart valves. The rationales for this hypothesis supplemented the design and use of a novel cyclic stretch bioreactor in development of engineered cardiac valve tissue.

In future studies, we will aim to both quantitatively characterize the structural (i.e., collagen fiber network morphology) and mechanical properties of cell-seeded, computationally designed tri-layered scaffolds, as well as evaluate said scaffolds and cell-seeded constructs under simulated physiologic conditions in organ level bioreactors. Further, we will aim to investigate tissue formation by valvular interstitial cells as well as clinically relevant mesenchymal stem cells. Collectively, this study provides the foundation for a more deterministic scaffold design and fabrication approach that can, in principal, be adapted to a variety of cardiovascular tissue repair and regeneration applications.
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