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Laser Microfabricated Poly(glycerol Sebacate) Scaffolds for Heart Valve Tissue Engineering

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Abstract

Microfabricated poly(glycerol sebacate) (PGS) scaffolds may be applicable to tissue engineering heart valve leaflets by virtue of their controllable microstructure, stiffness, and elasticity. In the current study, PGS scaffolds were computationally designed and microfabricated by laser ablation to match the anisotropy and peak tangent moduli of native bovine aortic heart valve leaflets. Finite element simulations predicted PGS curing conditions, scaffold pore shape, and strut width capable of matching the scaffold effective stiffnesses to the leaflet peak tangent moduli. Based on simulation predicted effective stiffnesses of 1.041 MPa and 0.208 MPa for the scaffold preferred (PD) and orthogonal, cross-preferred (XD) material directions, scaffolds with diamond-shaped pores were microfabricated by laser ablation of PGS 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 both 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 μ g/g wet weight) and DNA (17 ± 4 μ g/g wet weight). This study provides a basis for the computational design of biomimetic microfabricated PGS scaffolds for tissue engineered heart valves.

Keywords

Tissue Engineered; Heart Valve; Poly(glycerol sebacate); Biomimetic; Finite Element

INTRODUCTION

Advanced bioprosthetic and mechanical replacement heart valves, while functionally inferior to their healthy native counterparts, are both lifesaving and relatively long-lasting in adults suffering from valvular disease.¹ Such advances in valve replacement, however, have yet to be fully translated to neonates and young children suffering from congenital valvular lesions. Children may require as many of five reoperations prior to adulthood to accommodate increases in ventricular outflow tract diameter associated with somatic

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growth.² Toward improving the quality of life of these young patients, investigators are pursuing the development of a living, tissue engineered heart valve (TEHV).^{3,4}

Early TEHVs were fabricated *in vitro* by cultivating autologous, vascular-derived cells on nonwoven poly(glycolic acid) (PGA) scaffolds. First developed by Shinoka et al. in the laboratory of Dr. John E. Mayer, Jr. at Children's Hospital Boston,⁵ the potential of this prototypical TEHV paradigm has since been demonstrated in several large animal studies. Notably, Hoerstrup et al.⁶ demonstrated 5-month function of TEHVs as pulmonary valve replacements in growing lambs, correlating with functional maturation of the TEHV cellular phenotypes and extracellular matrix composition and organization, as demonstrated by Rabkin et al.⁷ Toward clinical translation, Sutherland et al.⁸ demonstrated long-term function of TEHV constructed by seeding similar nonwoven scaffolds with relatively noninvasively accessible bone marrow-derived mesenchymal stem cells. Recent advances by Gottlieb et al.⁹ further refined the original paradigm while underscoring one of its potential limitations: the high stiffness, inelasticity, and complex micromechanics^{10,11} of the needle-punched nonwoven scaffold and its component PGA and poly(L-lactic acid) (PLLA) fibers.

Toward designing scaffolds with improved elasticity and structural-mechanical anisotropy similar to native semilunar valve leaflets, Courtney et al.¹² introduced an electrospun poly(ester urethane urea) scaffold exhibiting a biaxial mechanical response similar to native valve leaflet tissue. Electrospinning, however, is an inherently stochastic process in which control of microstructural morphology is possible only in an average sense. Sales et al.¹³ first investigated the utility of poly(glycerol sebacate) (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. PGS was developed by Wang et al.¹⁴ in the Langer Lab at MIT and exhibits compliant, elastomeric mechanical properties tunable to those of native cardiovascular tissues^{15,16} and supportive of vascular elastogenesis^{17–19}. A potential limitation of PGS foam, however, is its relatively low stiffness (4–8 kPa) compared with native valve leaflet tissues and effectively random pore structure.¹³

By contrast, Engelmayer et al. demonstrated that excimer laser microfabricated PGS scaffolds comprised of geometrically well-defined 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.¹⁵ To facilitate scaffold design, Jean and Engelmayer developed periodic finite element models capable of retrospectively predicting the effective stiffnesses and anisotropy of accordion-like honeycomb PGS scaffolds,²⁰ as well as the influence of scaffold-mediated anisotropic collagen fibrillogenesis.²¹ Toward the important goal of developing a deterministically rendered and biomimetic TEHV, 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. To the best of the authors' knowledge, this is the first study to combine microfabrication and predictive modeling in the context of TEHV scaffold design.

MATERIALS AND METHODS

Native bovine heart valve leaflet tissue mechanical testing

Bovine hearts from 20–30 month old bulls (Black Angus breed) were kindly donated by Brennenman's Meat Market (Huntingdon, PA). 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). Aortic valve leaflets were dissected from each of two hearts, providing 3 leaflets each for cutting circumferential (CIRC) and radial (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 $\times$ 5 mm wide CIRC or 15 mm long $\times$ 5 mm wide RAD rectangular strips [Fig. 1(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. Each specimen (gauge length = 5 mm) was strained to failure at a displacement rate of 10 mm/min at room temperature and moist but not submerged. 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 (ϵ_f) were taken as the maximum stress and strain measured at the onset of failure, respectively. An anisotropy ratio r was calculated by dividing E_{CIRC} by E_{RAD} , providing an index of the directional dependence of the tensile mechanical response.

Scaffold geometry and computational modeling

2-D periodic finite element models were developed using Z-set software (Northwest Numerics and Modeling, Inc., Coventry, RI) as in our previous studies.^{20,21} The Young's modulus of the 160°C/12h PGS struts was prescribed to be $E_{PGS} = 2.1 \pm 0.05$ MPa (based on uniaxial tensile tests of PGS membranes (Fig. 2)). Specifically, simulations were conducted for a range of E_{PGS} values: 2.05 MPa (low), 2.1 MPa (mean), and 2.15 MPa (high). The scaffold was defined as a tessellation of diamond-shaped pores of the same size and same geometry (e.g., 1.7:1 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_I and v_{II} , are depicted in Figure 3 and were coded in C++ as a function of the inside strut length l , the strut width w , and the acute inside angle α :

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

$$v_{II} = (w+l)\sin\left(\frac{\alpha}{2}\right)x + (w+l)\cos\left(\frac{\alpha}{2}\right)y \quad (2)$$

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 Figure 3) along translation vectors t calculated from combinations of the two primary vectors of periodicity:

$$t = Av_1 + Bv_2 \quad (3)$$

As depicted in Figure 3, the central diamond had 4 first neighbors (i.e., having a common edge; demarcated by a blue line) and 4 second neighbors (i.e., having a common vertex; indicated by white dots). 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 Figure 3), (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 Z-set, the diamond unit cell (periodic computations) or tessellation (kinematic uniform boundary conditions) was meshed using either square or triangular elements (for simulations involving sharp or rounded corner pores, respectively) with a quadratic interpolation of the displacement (e.g., 2028 elements and 6189 nodes per diamond unit cell). Computations were conducted according to our previous studies.^{20,21}

PGS pre-polymer synthesis and curing

PGS pre-polymer was synthesized by adapting previously described methods.^{14,15} 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 10×10"; 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). High 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) membranes of cured PGS were adapted from previous studies.^{15,22} In brief, standard glass microscope slides (75 × 25 × 1mm; VWR) were first soaked overnight in 0.5 M NaOH (Sigma) to improve wettability, rinsed exhaustively in distilled water, and then coated with a thin layer of 90% (w/v) sucrose (Sigma) in distilled water using a custom-built spin-coater (3000 RPM; 30 s). Sucrose-coated slides were then baked in an oven (Model 1470; VWR) at 120°C for 18 h prior to use. To target a PGS membrane thickness of 250 μm, 0.5 g of PGS pre-polymer was melted and uniformly spread on a sucrose-coated slide using a stainless steel spatula. PGS pre-polymer coated slides were then cured in a vacuum oven (Model 1470; VWR) under high vacuum (< 50 mTorr; Model 1405; Welch Vacuum Technology) at 160°C for times ranging from 8–16 h to yield PGS with different mechanical properties.¹⁶ Following curing, PGS membranes were left adhered to sucrose-coated glass slides and stored in a dessicator at room temperature. Prior to use, PGS membranes 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,²³ and then rehydrated in distilled water prior to laser microablation or mechanical testing.

PGS scaffold fabrication by laser microablation

PGS scaffolds were microfabricated by adapting methods previously developed by Engelmayer et al.¹⁵ 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).^{24,25} 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,²⁶ 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 axis by short axis dimensions of the diamond shaped pores ablated through the PGS membrane (obtained via the 3:1.5 mm aperture) were determined in pilot experiments to be $\sim 240 \times 140$ microns (i.e., $\sim 1.7:1$, in contrast to the aperture aspect ratio of 2:1). Of note, in describing the directionally-dependent behaviors of the scaffold in a manner consistent with previous studies,^{11,15,20,21,24,25} herein the material direction parallel to the pore long axis is termed the preferred direction (PD), and the orthogonal material direction is termed the cross-preferred direction (XD). In order to pattern the desired diamond shaped pore tessellation, a custom program was developed using Microsoft Excel Visual Basic for Applications (VBA) to interface with the LSX-213 Digilaz II software (v3.1.0; CETAC, Inc.).²⁴ PGS membranes ($\sim 75 \times 25$ mm; ~ 250 μ m thick) were removed from distilled water and flattened out on a flat piece of black rubber that was pre-wetted using ~ 2 mL of 90% (w/v) sucrose in distilled water solution (to promote PGS adhesion to the rubber; PGS membranes tended to contract upon prolonged laser exposure). Laser parameter values used herein were: 50% energy level (i.e., yielding ~ 2.25 mJ per pulse), 20 Hz pulse frequency, 300 pulses per pore, and a defocus value of 1000 microns (i.e., positioning the laser focal point ~ 1000 microns below the top surface of the PGS membrane, which qualitatively yielded sharper features and reduced top-to-bottom pore tapering).

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) at a 20 kV accelerating voltage, $\sim 10.2 - 10.5$ mm working distance, and 0.60 Torr pressure level.

Image analysis

A representative scanning electron micrograph of a PGS scaffold ([Fig. 5(b)], 300x) was manually analyzed to measure the inside dimensions of the pore long (PD) and short (XD) axes, the radii of curvature of the inside PD and XD corners of the pores, and the strut width (ImageJ ver. 1.45s; National Institutes of Health, Bethesda, MD, USA²⁷). A total of 12 pores and struts were measured.

PGS membrane and PGS scaffold mechanical testing

Solid PGS membranes cured for different times (8, 12, and 16 h) at 160°C and laser ablated PGS scaffolds ($\sim 25 \times 5 \times 0.25$ mm) were mechanically tested in uniaxial tension as described above for bovine aortic valve leaflet tissues, with the following modifications. PGS membrane and PGS scaffold specimens were first aligned and affixed to a heavy paper tab using a hot glue gun as described previously.¹⁵ In contrast to the native leaflet tissues, the effective stiffness (E) of each PGS membrane and PGS scaffold specimen was determined by linear regression on the stress-strain data in the initial (i.e., 0–10%) region. The term “effective stiffness” utilized herein in a manner consistent with previous studies denotes the initial stiffness of the heterogeneous, but considered homogenous, PGS scaffold microstructure (i.e., consisting of intervening PGS and void spaces), and was applied to the essentially linear elastic, homogenous, isotropic PGS membranes for consistency.

Cell culture, scaffold seeding, and construct cultivation

Fetal Sprague-Dawley rat skin fibroblasts (27FR; ATCC # CRL-1213; ATCC, Manassas, VA) were utilized as a model, collagen-synthesizing cell type as used previously by

Engelmayr et al.²⁶ 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)). Prior to seeding, microfabricated PGS scaffolds ($\sim 5 \times 5 \times 0.25$ mm; $n=3$) were autoclave-sterilized (121°C/30 min) and soaked in complete medium for 6 days to help improve cell attachment.²⁸ The general seeding techniques have been described previously.^{15,26,29,30} Each scaffold was placed into an individual, sterile-vented 50 ml bioreactor tube (Product # 87050; TPP Techno Plastic Products AG, Trasadingen, Switzerland). Cell suspension (12 ml) was pipetted into each of the three bioreactor tubes yielding a seeding density of $\sim 10 \times 10^6$ cells/cm². The bioreactor 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, Inc., Plymouth, MN). 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.³¹

Laser scanning confocal microscopy

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.¹⁵ In brief, samples of cell-seeded scaffold were rinsed in HBSS and fixed overnight at 2–8°C in 10% neutral buffered formalin (Sigma), labeled with Alexa Fluor 488-phalloidin (Invitrogen) and coverslipped with Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) to counterstain cell nuclei. Samples were imaged with an 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.

Collagen and DNA assays

The DNA content of engineered tissue extracts was quantified using the PicoGreen dsDNA quantitation kit (Invitrogen) per the manufacturer's instructions as described previously^{15,29,30} using a Spectramax Gemini XS plate reader (Molecular Devices, Inc., Sunnyvale, CA). 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) as described previously.^{29,30}

Statistical analysis

Reported values represent mean $\pm$ standard error (at least $n = 3$ specimens). Comparisons between groups were made using the Student's *t*-test. Differences between groups were deemed statistically significant for *p*-values < 0.05 . Statistical analyses were performed using Microsoft Excel.

RESULTS

Native bovine aortic valve leaflet mechanical properties

Representative uniaxial tensile stress-strain curves for CIRC and RAD strips of native bovine aortic valve leaflet tissue are presented in Figure 1(b). In particular, the following mean $\pm$ standard error mechanical property values were measured (note that E_{CIRC} and

E_{RAD} values reported here for native bovine aortic valve leaflet tissues represent peak tangent moduli measured in the post-transition region of the stress-strain curve): $E_{CIRC} = 1.00 \pm 0.16$ MPa, $UTS_{CIRC} = 0.251 \pm 0.02$ MPa, and $\epsilon_{fCIRC} = 76 \pm 4$ %; $E_{RAD} = 0.26 \pm 0.03$ MPa, $UTS_{RAD} = 0.09 \pm 0.01$ MPa, and $\epsilon_{fRAD} = 106 \pm 8$ %; anisotropy ratio $E_{CIRC}/E_{RAD} = 3.90$.

PGS synthesis and mechanical properties

PGS pre-polymer was synthesized successfully (i.e., grossly yielded the expected appearance and soft waxy consistency upon cooling to room temperature). PGS pre-polymer 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,¹⁵ demonstrating a similar trend of increased E_{PGS} with increased curing time at constant temperature (Fig. 2). In particular, the following mean $\pm$ standard error mechanical property values were measured: $E_{PGS} = 1.15 \pm 0.06$ MPa, $UTS_{PGS} = 0.44 \pm 0.07$ MPa, and $\epsilon_{fPGS} = 54 \pm 7$ % (for 8h/160°C); $E_{PGS} = 2.1 \pm 0.05$ MPa, $UTS_{PGS} = 0.64 \pm 0.06$ MPa, and $\epsilon_{fPGS} = 34 \pm 3$ % (for 12h/160°C); $E_{PGS} = 2.47 \pm 0.02$ MPa, $UTS_{PGS} = 0.626 \pm 0.04$ MPa, and $\epsilon_{fPGS} = 28 \pm 2$ % (for 16h/160°C).

Finite element simulations

Based in particular on the measured anisotropy ratio of the native leaflet tissues (i.e., $E_{CIRC}/E_{RAD} = 3.90$), it was decided to focus initial finite element modeling efforts on simulating scaffolds fabricated using the 2:1 aspect ratio aperture (i.e., that yielding $\sim 1.7:1$ aspect ratio diamond-shaped pores in pilot experiments). Finite element simulations based on the 1.7:1 aspect ratio diamond-shaped pore (i.e., 240×140 micron pore inside dimensions), 50 micron wide struts (chosen based on our previous study¹⁵), and PGS cured for 12 h at 160°C (i.e., having an E_{PGS} value of 2.1 ± 0.05 MPa) were conducted [Fig. 4(a, b)]. Varying E_{PGS} based on the range of values measured, predicted scaffold effective stiffnesses ranged from: $E_{PD} = 0.992$ MPa and $E_{XD} = 0.198$ MPa (low; $E_{PGS} = 2.05$ MPa); $E_{PD} = 1.041$ MPa and $E_{XD} = 0.208$ MPa (mean; $E_{PGS} = 2.1$ MPa), and $E_{PD} = 1.103$ MPa and $E_{XD} = 0.220$ MPa (high; $E_{PGS} = 2.15$ MPa); predicted anisotropy ratio $E_{PD}/E_{XD} = 5$. Of note, for these idealized scaffolds with perfectly sharp inside pore corners, the maximum equivalent von Mises strains predicted to manifest within the PGS struts themselves (under the simulated application of 20% macroscopic strains to the scaffold [Fig. 4(a, b)]) were $\sim 137\%$ (PD) and $\sim 89\%$ (XD).

PGS scaffold fabrication, image analysis, and simulation

PGS scaffolds based on this design were microfabricated by 213 nm laser ablation. Representative scanning electron micrographs of the resultant scaffolds comprised of $\sim 1.7:1$ aspect ratio diamond-shaped pores and ~ 50 micron wide struts are presented at 100x and 300x magnifications [Fig. 5(a, b)]. Image analysis of the 300x scanning electron micrograph [Fig. 5(b)] yielded measurements of the actual pore and strut dimensions for comparison with expected values (Table 1). Based on measured pore and strut dimensions, additional finite element simulations were conducted [Fig. 4(c, d)]; in particular, to determine the sensitivity of scaffold effective stiffnesses to the experimentally observed radii of curvature of the inside corners of the pores. Varying E_{PGS} based on the range of values measured, predicted effective stiffnesses for scaffolds based on measured dimensions reported in Table 1 ranged from: $E_{PD} = 0.887$ MPa and $E_{XD} = 0.154$ MPa (low; $E_{PGS} = 2.05$ MPa); $E_{PD} = 0.908$ MPa and $E_{XD} = 0.158$ MPa (mean; $E_{PGS} = 2.1$ MPa), and $E_{PD} = 0.93$ MPa and $E_{XD} = 0.162$ MPa (high; $E_{PGS} = 2.15$ MPa); predicted anisotropy ratio $E_{PD}/E_{XD} = 5.75$. For scaffolds based on measured dimensions reported in Table 1, the maximum equivalent von Mises strains predicted to manifest within the PGS struts themselves (under the simulated

application of 20% macroscopic strains to the scaffold with rounded inside corners) were ~70% (PD) and ~46% (XD).

PGS scaffold mechanical properties

Representative uniaxial tensile stress-strain plots for separate PD and XD specimens of these scaffold materials are presented in Figure 6. In particular, the following mean $\pm$ standard error mechanical property values were measured for PGS scaffolds comprised of ~1.7:1 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 $\epsilon_{fPD} = 62 \pm 14$ %; $E_{XD} = 0.21 \pm 0.03$ MPa, $UTS_{XD} = 0.14 \pm 0.02$ MPa, and $\epsilon_{fXD} = 73 \pm 12$ %; measured anisotropy ratio $E_{PD}/E_{XD} = 3.95$.

Cell-seeded PGS scaffold DNA and collagen contents

Representative phase contrast light micrographs of cell-seeded scaffolds following 3 weeks static *in vitro* cultivation are presented at 100x [Fig. 7(a)] and 200x [Fig. 7(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 Figure 7c. Biochemical assays for collagen and DNA yielded mean $\pm$ standard error concentrations of 736 ± 193 μ g/g wet weight (for collagen) and 17 ± 4 μ g/g wet weight (for DNA).

DISCUSSION

Native semilunar heart valve leaflets are anatomically complex,³² belying what upon cursory inspection appear to be simple physiologic check valves. Indeed, recent studies have begun to demonstrate computationally^{33–35} what has long been suspected heuristically: that the physiologic mechanical function of native heart valve leaflets not only derives from, but explicitly depends upon, their specialized collagenous microstructure. Indeed, recent advances in combined imaging and micromechanical analysis are shedding light on how anisotropic strains are transmitted to the leaflets' interstitial³⁶ and endothelial³⁷ cellular constituents. Toward mimicking the microstructure and associated function of native leaflets, microfabrication techniques (e.g., 3-D printing³⁸) and mathematical modeling (e.g., computational models,^{35,39} analytical models^{10,11}) have each been independently applied in the context of TEHV scaffold design.

Finite element predicted versus experimentally measured effective stiffnesses

In the current study, initial finite element model predictions, based on the expected, idealized scaffold geometry, overestimated the scaffold effective stiffness measured in the PD direction by ~25% (1.041 MPa predicted versus 0.83 ± 0.13 MPa measured) and very closely matched the scaffold effective stiffness measured in the XD direction (0.208 MPa predicted versus 0.21 ± 0.03 MPa measured). Deviations between the computational and experimental results can be attributed to a combination of factors, including variability in E_{PGS} , as well as subtle differences in the microfabricated scaffold geometry (e.g., relatively rounded corners) compared with the idealized finite element model (e.g., relatively sharp corners). Based on experimentally measured pore and strut dimensions quantified by image analysis (Table 1), we undertook additional finite element simulations to investigate the influence of the curvature observed within the diamond-shaped pore inside corners. Of note, E_{PD} predicted by the image analysis-based finite element model (0.908 MPa for an E_{PGS} of 2.1 MPa) was well within the experimentally measured range, while the corresponding value of E_{XD} (0.158 MPa for an E_{PGS} of 2.1 MPa) deviated further from the experimentally measured value than the value of E_{XD} predicted from the initial, idealized model. Nevertheless, despite minor deviations, the experimentally measured E_{PD} and E_{XD} values

were bracketed within the collective range of finite element predictions generated based on the experimentally measured range of E_{PGS} , the idealized scaffold geometry, and the image analysis-measured scaffold geometry. Further, both the model predicted and experimentally measured effective stiffnesses were in relatively close agreement with the corresponding experimentally measured peak tangent moduli of the native bovine aortic valve leaflet tissues ($E_{CIRC} = 1.00 \pm 0.16$ MPa and $E_{RAD} = 0.26 \pm 0.03$ MPa). Thus, to the best of the authors' knowledge, ours is the first study to demonstrate the computational design and microfabrication of a scaffold closely mimicking; albeit with limitations, as described below; aspects of the mechanics of a native heart valve leaflet.

Native bovine aortic heart valve leaflet mechanical testing and properties

Notably, bovine aortic valve leaflet tissues were found to be considerably less stiff than their porcine (e.g., 7.78 ± 1.7 MPa CIRC; 1.28 ± 0.34 MPa RAD⁴⁰) and adult human (e.g., 15.34 ± 3.84 MPa CIRC; 1.98 ± 0.15 MPa RAD⁴¹) counterparts. We attribute these deviations in part to the greater thickness of the bovine aortic valve leaflet tissues (~1.5 mm) compared with that reported for porcine (e.g., 0.5 mm⁴²) and adult human (e.g., 0.605 ± 0.196 mm⁴¹) aortic valve leaflet tissues. In contrast to porcine and adult human semilunar heart valve leaflets, comparatively little data has been reported on the tensile mechanical properties of fresh native bovine aortic valve leaflets; to the best of the authors' knowledge, the only study explicitly reporting tensile mechanical properties for fresh bovine aortic valve leaflets is Firor and Sharma.⁴³ However, that study focused on reporting tensile loads, and did not report the leaflet thicknesses, nor the age or breed of the bovines (other than being adult steers), precluding a direct comparison to the current work. In our laboratory, similar tensile mechanical properties were obtained for younger black angus bulls (i.e., 14–15 months) utilizing a different uniaxial tensile mechanical tester (unpublished observations), suggesting that the tensile stress-strain behavior of fresh bovine aortic valve leaflets is indeed distinct from that of porcine and human leaflets. Of note, a recent study by Aldous et al.⁴⁴ investigated all four of the bovine heart valves (24–30 month old adult steers) by hydrothermal isometric tension (HIT) testing, observing differences in the degree and type of collagen cross-linking among the four valves. Further, the unique HIT testing approach applied by Aldous et al. highlights the manifold and multi-scale approaches by which the mechanical properties of soft tissues can be characterized, including, for example, microindentation.⁴⁵

Limitations of uniaxial tensile testing for heart valve leaflet tissue characterization

From the perspective of mechanical testing, a potential limitation of our study was that we relied exclusively on uniaxial tensile testing. Billiar and Sacks comprehensively addressed the biaxial mechanical response of both fresh and glutaraldehyde-fixed porcine aortic valve leaflet tissues,⁴⁶ developing a structural-based constitutive model capable of predicting the full planar biaxial response from knowledge of the underlying collagen fiber orientations, including in-plane shear.⁴⁷ More recently, Liao et al.⁴⁸ demonstrated molecular level rearrangements in these porcine aortic valve tissues under biaxial loading. While uniaxial tensile testing is capable of elucidating gross directional differences in tensile mechanical properties, biaxial data spanning multiple loading protocols is generally considered essential to developing constitutive models capable of fully describing the heterogeneous, multi-axial deformations that heart valve leaflets undergo physiologically *in vivo*. In future studies, it will be instructive to evaluate microfabricated PGS scaffolds under biaxial loading, as such studies would be expected to yield a better understanding of scaffold mechanical behavior under simulated *in vivo* loading conditions.

Limitations of microfabricated PGS scaffold linear elastic properties

Another limitation of the microfabricated PGS scaffolds demonstrated herein is their essentially linear elastic behavior (Fig. 6), which cannot, with the current diamond-shaped pore geometry and 50 micron strut width, fully mimic the highly nonlinear stress-strain response of native heart valve leaflet tissues [Fig. 1(b)]. In light of this limitation, in the current study we chose to target matching the peak tangent modulus, rather than the initial modulus, of the native bovine aortic valve leaflet. The rationale for matching the peak tangent modulus was that upon implantation (neglecting the contribution of *de novo* synthesized extracellular matrix), the scaffold would need to be capable of withstanding peak loads associated with valve closure during diastole. Supporting this rationale, the ultimate tensile strength of the scaffold exceeded that measured for the native bovine aortic valve leaflet by ~49% in the PD direction ($UTS_{PD} = 0.4 \pm 0.1$ MPa versus $UTS_{CIRC} = 0.251 \pm 0.02$ MPa) and by ~55% in the XD direction ($UTS_{XD} = 0.14 \pm 0.02$ MPa versus $UTS_{RAD} = 0.09 \pm 0.01$ MPa).

Given the ability to custom fabricate alumina ceramic masks with arbitrary shaped apertures, the laser microablation approach could potentially be utilized to fabricate PGS scaffolds with microstructures intrinsically manifesting nonlinear tensile mechanical properties (e.g., comprised of thin, undulated structural elements; similar to, albeit on smaller length scales, previous accordion-like honeycomb PGS scaffolds¹⁵). Of note, Fozdar et al.⁴⁹ previously utilized digital micro-mirror projection printing to photo-crosslink acrylated poly(ethylene glycol) into scaffolds exhibiting unique microstructures with tunable, negative Poisson's ratios, and likely amenable to yielding nonlinear tensile properties.

A key consideration, however, in obtaining a fully nonlinear mechanical response from such prismatic honeycomb scaffolds is the ratio of the strut width to the pore inside strut length, as it is this ratio that dictates whether the initial mechanical response of the scaffold will be dominated by strut bending or strut axial loading. As demonstrated in the seminal work of Gibson et al.⁵⁰, for compressive loading of hexagonal honeycomb structures, a strut width to inside strut length ratio less than 0.25 will yield bending-dominated strut deformation in the low stress-strain regime. In the case of our diamond-shaped scaffolds with 50 micron wide struts and an inside strut length of ~139 microns, the corresponding ratio is ~0.36, suggestive of axial loading-dominated strut deformation throughout the entire stress-strain response, up to the point of failure. Barring relative motion (e.g., rotation) of struts with respect to one another, a nonlinear tensile stress-strain response reminiscent of soft collagenous tissues would not be anticipated of the scaffold without a progressive transition from strut bending to strut axial loading with increasing strains. PGS has been shown to degrade principally by lipase-mediated surface hydrolysis *in vivo*, with an ~14% mass loss and ~8% mechanical strength loss per week observed over a period of 5 weeks subcutaneous implantation.^{51,52} Surface hydrolysis is thus expected to yield progressively thinner struts during *in vivo* implantation of a microfabricated PGS scaffold; it is anticipated that PGS scaffolds would begin to exhibit a nonlinear tensile mechanical response following either lipase-mediated surface hydrolysis *in vitro* or implantation *in vivo*. Alternatively, PGS scaffolds could be directly microfabricated with thinner struts; pilot experiments suggest that 20 microns likely represents a lower limit on strut width, due to the impact of scattered laser energy on the PGS struts. Interestingly, we previously predicted that microfabricated PGS scaffolds comprised of accordion-like honeycomb pores would incur not only changes in stiffness magnitude, but also changes in anisotropy, with the progressively diminishing strut widths characteristic of surface hydrolysis-mediated degradation.²⁰ Such changes in anisotropy are related to both changes in the dominant mode of strut deformation, as well as associated changes in PGS volume fraction, and suggest potential opportunities for more complex scaffold functionalities.

Cell-seeded scaffold composition and organization

Of note, the mean collagen concentration measured in the current study for rat skin fibroblast-seeded microfabricated PGS scaffolds ($736 \pm 193 \mu\text{g/g}$ wet weight) was comparable to that measured previously for nonwoven scaffolds cultivated statically for 3 weeks with ovine vascular-derived smooth muscle cells ($546 \pm 111 \mu\text{g/g}$ wet weight²⁹) and ovine bone marrow-derived mesenchymal stem cells ($422 \pm 98 \mu\text{g/g}$ wet weight³⁰). As the structural elements of the PGS scaffold are expected to progressively diminish in dimension, primarily by surface hydrolysis,⁵¹ the mechanical behavior of the engineered tissue is expected to progressively approach that of the cell-secreted collagenous extracellular matrix.¹⁰

Toward promoting native-like collagen fiber alignment in the context of TEHV based on microfabricated scaffolds, Engelmayr et al.²⁶ demonstrated a positive correlation between the aspect ratio of microfabricated rectangular pores and collagen fiber alignment in a non-degradable scaffold material, as well as noted advantages of diamond-shaped pores in promoting more reproducible cell and collagen alignment. 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. 7(c)], cell and collagen fiber alignment were not explicitly quantified (as for microfabricated PGS scaffolds using a Fast Fourier Transform (FFT)-based method¹⁵). Because cells were seeded at a high density (i.e., $\sim 10 \times 10^6$ cells/cm²) resulting in relatively rapid aggregation and filling of pores, the growth front-mediated orientation effect (preferential along the pore long axis) observed previously²⁶ in similarly shaped pores seeded with the same fibroblasts at a low density (i.e., $\sim 7.5 \times 10^5$ cells/cm²) was not explicitly expected. Future studies will aim to systematically quantify relationships between scaffold pore geometry and cell orienting capacity.

Biomechanics and mechanobiology in heart valve tissue engineering

Heart valve leaflets and their constituent interstitial cells function normally in a mechanically dynamic state of homeostasis;⁵³ Huang et al. demonstrated that the deformation of aortic valve interstitial cells (VICs) *in situ* within leaflet tissues correlate positively, though in a complex, nonlinear fashion, with diastolic transvalvular pressure and associated tissue strains.⁵⁴ In turn, the VICs themselves appear to be mechanically tuned to their particular mechanical environment; Fisher et al.⁵⁵ recently demonstrated that VICs aggregate and calcify to a greater degree when subjected to strain, and Wyss et al.⁵⁶ demonstrated pathological differentiation of VICs associated with increased substrate stiffness. Highlighting the importance of these lines of inquiry, a culture system reported by Throm Quinlan et al.⁵⁷ was utilized to investigate the coupled effects of stretch and substrate stiffness on VICs, and Warnock et al.⁵⁸ recently demonstrated *in situ* pressure-mediated changes in inflammatory gene expression by VICs.

Hoerstrup et al. was the first to demonstrate the benefit of simulating the native dynamic mechanical environment in the *in vitro* bioreactor conditioning of TEHV.^{6,59} Cyclic mechanical loading, in particular large strains, as demonstrated by Mol et al.,⁶⁰ has been shown to promote native valve-like collagen fiber orientation in TEHV constructs based on relatively inelastic nonwoven scaffolds.⁶¹ It is thus expected that bioreactor application of cyclic mechanical loading to TEHV materials^{29,30,62} based on microfabricated, elastomeric PGS scaffolds will likely enhance preferential cell and collagen alignment. Of note, the feasibility of cyclically stretching microfabricated elastomeric PGS scaffolds has been demonstrated previously via *in vitro* fatigue studies in the absence of cells.¹⁵ 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.⁶³ In evaluating the potential benefits of bioreactor-mediated conditioning of TEHV based on clinically relevant stem and progenitor cells, it will likewise be important to seek detailed molecular (e.g., gene) level evidence of valvulogenic cell differentiation, such as established by Latif et al.⁶⁴ and under investigation in the context of TEHV by Emani et al.⁶⁵ Finally, while the current study emphasized the modeling and microfabrication of TEHV scaffolds based on the bioresorbable elastomer PGS, a variety of other promising scaffold materials-of-construction,^{66–69} microfabrication approaches,^{70–72} seeding,^{73,74} and minimally invasive delivery strategies⁷⁵ are currently under development in the context of TEHV.

CONCLUSION

PGS scaffolds for TEHV were computationally designed and microfabricated by laser ablation to mimic the peak tangent moduli and anisotropy of native bovine aortic heart valve leaflet tissues. 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 PGS scaffolds, as well as evaluate said scaffolds and cell-seeded constructs under simulated physiologic conditions in bioreactors. Further, we will aim to investigate tissue formation by VICs 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 tissue repair and regeneration applications.

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Figure 1.

Schematic illustrating the preparation of circumferential (CIRC) and radial (RAD) uniaxial tensile mechanical test specimens from bovine aortic valve leaflets (A). 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). Representative uniaxial tensile stress-strain curves for CIRC and RAD stretched specimens of bovine aortic valve leaflet tissue (B). Associated representative peak tangent moduli (E_{CIRC} and E_{RAD}) 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.

Figure 2.

Effective stiffnesses (E; equivalent to Young's moduli) measured for solid PGS membranes cured for different times (8, 12, and 16 hours) at 160°C under high (< 50 mTorr) vacuum. For comparison, results obtained previously under equivalent curing conditions by Engelmayer et al.¹⁵ are presented side-by-side with results obtained in the current study. Note the similar trend of increased E_{PGS} with increased curing time at constant temperature.

Figure 3.

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 v_I and v_{II} used in generating the set of unit cell coordinates for building the tessellation are indicated.

Figure 4.

Representative 2-D periodic finite element simulations of the diamond-shaped unit cell deforming in response to an applied strain of 20% in the scaffold preferred (PD; A, C) and orthogonal, cross-preferred (XD; B, D) material directions. Expected, idealized pore geometries (A, B) are compared side-by-side with pore geometries measured manually by image analysis of representative scanning electron micrographs (C, D). Note that the red wire-frames indicates the undeformed unit cell shape. Color scale bar indicates the magnitude of equivalent von Mises strain (red = higher; blue = lower).

Figure 5. Representative scanning electron micrographs at 100x (A) and 300x (B) magnification of microfabricated PGS scaffolds comprised of ~1.7:1 aspect ratio diamond-shaped pores. Scaffold PD and XD material directions are indicated.

Figure 6.

Representative uniaxial tensile stress-strain curves for specimens of microfabricated PGS scaffold (~1.7:1 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.

Figure 7.

Representative phase contrast light micrographs (100x, A; 200x; B) 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) (250x, C). Scale bar = 200 microns.

Table 1

Expected (based on the idealized scaffold geometry and pilot experiments) and measured values for PGS scaffolds microfabricated using a 2:1 aspect ratio diamond-shaped aperture. Expected values refer to typical values observed in pilot experiments; in particular, it was noted in pilot experiments that pores microablated using the 2:1 aspect ratio diamond-shaped aperture tended toward 1.7:1 aspect ratio. Measured values were quantified manually by image analysis using ImageJ software²⁷.

	Pore Axis Length (μm)	Pore Inside Radius of Curvature (μm)	Scaffold Strut Width (μm)
PD_{Expected}	240	0	50
PD_{Measured}	242.8 ± 1.1	21.5 ± 0.5	45.2 ± 0.8
XD_{Expected}	140	0	50
XD_{Measured}	146.5 ± 0.9	39.0 ± 1.2	45.2 ± 0.8