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Tri-layered elastomeric scaffolds for engineering heart valve leaflets

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ABSTRACT

Tissue engineered heart valves (TEHVs) that can grow and remodel have the potential to serve as permanent replacements of the current non-viable prosthetic valves particularly for pediatric patients. A major challenge in designing functional TEHVs is to mimic both structural and anisotropic mechanical characteristics of the native valve leaflets. To establish a more biomimetic model of TEHV, we fabricated tri-layered scaffolds by combining electrospinning and microfabrication techniques. These constructs were fabricated by assembling microfabricated poly(glycerol sebacate) (PGS) and fibrous PGS/poly(-caprolactone) (PCL) electrospun sheets to develop elastic scaffolds with tunable anisotropic mechanical properties similar to the mechanical characteristics of the native heart valves. The engineered scaffolds supported the growth of valvular interstitial cells (VICs) and mesenchymal stem cells (MSCs) within the 3D structure and promoted the deposition of heart valve extracellular matrix (ECM). MSCs were also organized and aligned along the anisotropic axes of the engineered tri-layered scaffolds. In addition, the fabricated constructs opened and closed properly in an *ex vivo* model of porcine heart valve leaflet tissue replacement. The engineered tri-layered scaffolds have the potential for successful translation towards TEHV replacements.

1. Introduction

Current approaches for heart valve replacements including advanced bioprosthetic and mechanical heart valves are lifesaving in adults suffering from valvular disease. However, there are several limitations associated with currently available heart valve prostheses. Mechanical valves are thrombogenic and require patients to undergo lifelong anticoagulation therapies [1–4]. Bioprosthetic heart valves have limited durability because of their susceptibility to degradation and calcification [3,5]. An important additional drawback for pediatric patient with valvular diseases is the inability of these replacement valves to grow *in vivo* with the patients, which may result in multiple operations. Autologous tissue engineered heart valves (TEHVs) aim to overcome these limitations by creating living, non-cytotoxic, mechanically analogous heart valve replacements that are able to grow and remodel with the patient [6–12].

Heart valves primarily consist of valvular interstitial cells (VICs) that are surrounded by an endothelial monolayer [13]. The extracellular matrix (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*, with collagen, glycosaminoglycans (GAGs) and elastin

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representing the predominant ECM component in these three respective layers [13]. This intricate ECM layered architecture dictates the anisotropic mechanical characteristics of valve leaflets [14–17]. More specifically, leaflet mechanical stiffness and elasticity are attributed to the surface fibrous layers; *zona fibrosa* and *zona ventricularis* [18]. Since valve leaflet function is dependent on this structure, ideally, engineered scaffolds should (a) resemble the native tissue microstructure [14,19], (b) match native mechanical properties and retain anisotropy [16,20], (c) have elastic characteristics in deformation similar to native tissue [21,22], and finally (d) possess a controlled degradation rate that preserves structural integrity while providing support for adequate tissue formation [23] but ultimately do not prevent tissue growth.

A typical TEHV approach is to seed natural [24,25] or synthetic scaffolds with cells [7,23,26–29], culture them in static *in vitro* environments or in bioreactors simulating tissue growth with physiological hemodynamics [30–33], and then implant the cell-seeded constructs *in vivo* [7,8,11,30]. Various cell sources such as vascular-derived smooth muscle cells [33], MSCs [34], VICs [23,29,35], and fibroblasts [28] have been utilized to engineer functional TEHV. Previous studies have aimed to create scaffolds, which mimic the structural complexity of the native tissues and provide an appropriate environment for cellular growth and *in vitro* ECM generation [7,8,24,26,28,29]. However, some of the drawbacks associated with these engineered scaffolds have prevented them from successful translation to *in vivo* models. These limitations include non-physiological stiffness (e.g. non-woven scaffolds) [7,32,36], lack of anisotropic characteristics (e.g. homogenous fibrin gels or fibrous scaffolds comprising of random fibers) [10,23,25,26,37], inappropriate micro-architecture (e.g. non-fibrous scaffolds) [27–29], plastic deformation under stress [23,38] and a lack of suturability (e.g. hydrogel based scaffolds and microfabricated scaffolds) [24,39]. Biodegradable elastomers have been synthesized for cardiovascular tissue engineering for their potential to withstand physiological cyclic loads and viscoelastic properties resembling native tissues [22,40–46]. Among these, poly(glycerol sebacate) (PGS) has been employed extensively due to its elasticity, biocompatibility and controlled degradation rate [28,29,47,48]. However, the elastic modulus of PGS varies between 0.18 and 1.5 MPa depending on curing conditions (time and temperature) and scaffold structure [28,48,49], which is considerably lower than the native leaflet stiffness (4–8 MPa) [18,29].

Previously, fibroblast and VIC seeded microfabricated PGS scaffolds, with diamond-shaped pores, provided adequate anisotropy matching native leaflet properties while supporting tissue formation and ECM deposition [28,29]. However, these constructs lacked a fibrous structure, and the existence of micropores limited their suturability, impeding their applications in *in vivo* studies. To resemble the fibrous structure of native tissues, we recently fabricated electrospun fibrous PGS/poly(caprolactone) (PCL) scaffolds with anisotropic and tunable mechanical properties, including aligned fibers that matched the stiffness of native tissues [38,50]. However, the stress–strain curves for electrospun PGS/PCL scaffolds demonstrated large creep deformation. In addition, the small pores in these scaffolds (pore size <8 μm) prevented cell migration and ECM deposition through their 3D structures, which limited the formation of 3D tissue constructs [38,50].

Here we aimed to fabricate biomimetic tri-layered elastic scaffold with anisotropic properties similar to the structure and mechanics of the native leaflets. A semi-automated layer-by-layer assembly was applied to fabricate this 3D construct with tunable mechanical properties. We hypothesized that combining PGS/PCL microfibers and microfabricated PGS in a tri-layered construct would provide both elasticity and anisotropy that mimics the structural and mechanical properties of native leaflets while simultaneously supporting controlled cellular growth and tissue formation with controlled architecture. This approach could have the potential for successful translation towards a TEHV replacement.

2. Materials and methods

2.1. Fabrication and assembly of tri-layered scaffolds

2.1.1. Polymer synthesis

PGS pre-polymer was synthesized through polycondensation of glycerol and sebacic acid (1:1 molar ration) by using previously described procedures by Wang et al. [43]. Briefly, sebacic acid and glycerol with 1:1 molar ratio were reacted at 120 °C in high vacuum ($\sim$6.5 Pa) for 24 h to synthesize PGS pre-polymer.

2.1.2. PGS micromolding

The fabrication process used to design the PGS scaffolds, consisting of 2:1 aspect ratio diamond shape pores with approximately 75 μm-thick struts, was previously described in detail [29]. The mold was made from an ultra-high temperature machinable glass–mica ceramic sheet (0.5″ thick, 2″ × 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 h. A razor blade was used to cut the fabricated ceramic part away from the bottom sheet to release the polymer scaffold with a 300 μm thick from the ceramic sheet. 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)) (Fig. 1A).

2.1.3. Electrospinning and layer by layer assembly

A directional electrospinning system was employed to spin the pre-polymers into sheets. The pre-polymer mixture was pushed from a syringe pump at a flow rate of 2 ml/h 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 (Fig. 1B). The fabricated construct resembled the native leaflets tri-layered structure shown in Fig. 1C.

2.2. Valve leaflet dissection and VICs/MSCs isolation

Leaflets were aseptically excised from fresh sheep hearts, obtained from the Animal Research Facility (*ARCH*) in Children's 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 by collagenase digestion [29]. Briefly, the leaflets were wiped with sterile gauze to remove the valvular endothelial cells. VICs were then 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 h. The digested tissues were 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).

2.2.1. Cell seeding

In preparation for cell seeding, scaffolds were first sterilized by soaking in 70% (v/v) 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