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The mechanically enhanced phase separation of sprayed polyurethane scaffolds and their effect on the alignment of fibroblasts

James P. Kennedy, Sean P. McCandless, Richard A. Lasher, Robert W. Hitchcock*

Department of Bioengineering, University of Utah, 20 S 2030 E, Rm 108 Salt Lake City, UT 84112, USA

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ABSTRACT

This paper reports a method to fabricate anisotropic scaffolds of tunable porosity and mechanical properties. Scaffolds were fabricated using a computer controlled sprayed phase separation technique. Following fabrication, the sheets were elongated 0, 35 or 70% of their original length to induce varying degrees of scaffold alignment and anisotropy. The nonsolvent used in the phase separation was shown to affect porosity and the elastic modulus. Mouse embryo NIH-3T3 fibroblasts were cultured on the scaffolds to investigate cell response to the anisotropy of the scaffold. A 2D FFT method was used to quantify cellular alignment. Cells were shown to align themselves with the scaffold. This sheet-like scaffold material can be used in single plys or can be laminated to form porous 3D composite scaffolds.

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1. Introduction

Tissue engineering and regenerative medicine have the potential to develop novel biosynthetic materials for improved treatment, maintenance, and regeneration of diseased or damaged tissue. Development of materials that utilize tissue engineering strategies requires design considerations such as cell type, seeding and attachment, as well as molecular signals, and macromolecular matrix in order to develop constructs that improve or replace function of natural tissue [1–3]. Many types of engineered tissue rely on a provisional or permanent scaffold to generate a three-dimensional framework for cell attachment and tissue organization. Both natural and synthetic scaffold materials are used in tissue engineering [4,5]. Synthetically derived cell scaffolds can be permanent or degradable and facilitate expression and organization of the extracellular matrix (ECM). Architectural cues in these scaffolds have been shown to affect the morphology, organization, and phenotypic expression of cells in vitro [6–8]. In order to develop effective implants, the tissue engineer needs to be able to specify and tune the scaffold's morphological features for different applications. In addition, scaffold architecture must be designed to provide cues for cellular organization and form the basis for engineered tissue constructs that mimic tissue specific organization and physical properties.

Cardiac tissue is an example of highly structured tissue that relies on cellular organization for its function [9,10]. Cell scaffold materials can help cardiac tissue development by: (1) providing cues that induce alignment of cardiac myocytes, (2) allowing sufficient nutrient and cell infiltration necessary to form a 3D tissue construct, (3) modulating the cell type distribution of co-cultures, and (4) mimicking anisotropic mechanical stiffness of the heart. Scaffolds used for cardiac tissue engineering applications require development of design specifications that include scaffold alignment, structure, porosity, and stiffness all of which will influence cellular development, overall tissue organization, and bioreactor integration [11–13].

Various methods have been employed to fabricate scaffolds of varying porosity and anisotropy. For example, microfabrication techniques have been used to fabricate scaffolds with aligned structure [14,15]. Electrospinning methods have been employed with post process elongation to produce anisotropic fibrous scaffold architecture [16]. In order to create scaffolds that allow adequate nutrient and oxygen diffusion, methods such as 3D fiber deposition, sacrificial fiber electrospinning, and phase separation have been utilized to generate scaffolds of controlled porosity [17–20]. Spray phase separation (SPS) is method for creating scaffolds with control over alignment, porosity, and stiffness; however this method has not been directly applied for cardiac tissue scaffolds [19].

SPS fabricated scaffolds are produced using a method that simultaneously sprays a polymer solution and a nonsolvent onto the surface. The nonsolvent mixes with the solvent and the

* Corresponding author.
E-mail address: r.hitchcock@utah.edu (R.W. Hitchcock).

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polymer causing the polymer to precipitate. Some groups have used SPS methods to fabricate materials of varying porosity for drug delivery devices [21] and vascular graft materials [19,22]. SPS fabrication is a promising method for controlling scaffolds properties such as alignment, porosity, stiffness, and anisotropy, which are key features for directing cellular development, overall tissue organization, and bioreactor integration [11–13].

We hypothesized that post spray elongation of SPS scaffolds would generate scaffold microstructure alignment and in turn induce cellular alignment. Furthermore, we hypothesized that the nonsolvent (NS) EtOH concentration would affect scaffold porosity. In addition, we hypothesize that this material may be laminated into thick scaffold material. Here we report an SPS method for fabricating polyurethane scaffolds for tissue engineering applications.

2. Materials and methods

2.1. Scaffold fabrication

Scaffolds were fabricated using an SPS method. A 4% polyurethane solution was prepared by dissolving Tecoflex SG80 polyether polyurethane (Lubrizol Advanced Materials Inc. Cleveland, OH) in dimethylacetamide (DMAc) (Sigma Aldrich, St. Louis, MO). Polyether polyurethane was selected because of its known biocompatibility, ease of processing, and extensive use in the past for scaffold materials [23–25]. The polymer solution was sealed in glass storage containers prior to use and used immediately after opening to ensure that minimal solvent was lost through evaporation. Deionized water (referred to as 0% ethanol), 50% EtOH, and 70% EtOH solutions were used as the nonsolvent for precipitation.

Spray nozzles (Excel ES4, Porter Cable, Jackson, TN) were mounted onto a custom crossbar attached to the spindle head of a computer-controlled desktop milling machine (MaxNC 12, MAXNC, Gilbert AZ) to provide X–Y–Z control of spray pattern. Custom G-code was used to move the spray head and control the spray pattern and spray time. The spray nozzles traversed the substrate at a distance of 20 cm and a speed of 0.85 cm/s in a serpentine pattern yielding a total spray time of 2.5 min.

Aluminum frames were designed to facilitate manipulation and mechanical alignment of the scaffold material during and after spraying. The frames were fabricated from 0.32 cm thick 6016-T6 aluminum (McMAster-Carr, Princeton NJ) with an outside dimension of 5.3 cm × 3.7 cm and an inside dimension of 2.86 cm × 2.54 cm. Stainless steel hypodermic tubing sliders (Small Parts Inc., Miramar, FL) were assembled and attached to frames with UV cure adhesive to facilitate elongation and constrain the maximum elongation to 3.86 cm (35% elongation) or 4.86 cm (70% elongation). Frames without sliders were used to make scaffolds with no in-process mechanical alignment. Stainless steel screens (Type 316 Mesh #60, Small Parts Inc.) were attached to the opposite ends of the frame to provide a rigid, yet porous surface for scaffold adherence. A sheet of 0.32 cm thick aluminum (McMaster-Carr) was coated with silicone (VST 50 silicone elastomer, Factor II Inc., Lakeside, AZ) to prevent the scaffold from sticking to the aluminum. Three frames (one of 0%, 35% and 70% elongation) were clamped to this silicone coated aluminum backing which was subsequently placed inside the spray chamber.

The polymer solution and nonsolvent were sprayed simultaneously onto the frames at a pressure of 40 psi. Spray rates were calculated by measuring the mass loss of the spray reservoir after a 30 s test spray. Spray rates of the polymer solution and the nonsolvent were adjusted on the spray nozzle to 7.5 ± 0.5 g/min and 45.0 ± 5.0 g/min respectively to achieve uniform scaffold structure.

After the spray process was completed, scaffolds were immediately rinsed with gently flowing DI water for 1 min. The frames were then removed from the aluminum backing, and scaffolds were elongated 0%, 35% or 70% of their original length and allowed to dry for 24 h in the stretched conformation. After the scaffolds were dry, they were removed from the frames for testing.

2.2. Mechanical testing

Mechanical properties were measured in the direction aligned with the in-process elongation (preferred direction) and perpendicular (transverse) to this direction. A sample was removed from the center of each scaffold for mechanical testing ($n = 3$). The samples used for longitudinal testing measured 20.0 mm × 5.0 mm and the samples used for transverse testing measured 5.0 mm × 1.5 mm. These sizes were chosen based on the physical limitations of the scaffold and frame configuration. Samples were tested on an Instron 3342 (Instron, Norwood MA) with a 50 N load cell. A gauge length of 10 mm was used for longitudinal testing, while a gauge length of 3 mm was used for transverse testing. The samples were tested to 40% strain at a speed of 20 mm/min. The effective stress for each sample was calculated by dividing the force by the overall cross section. From the effective stress and strain data an effective modulus of elasticity was calculated in both the preferred and the transverse direction. Anisotropy is reported as the ratio of the effective modulus in the preferred direction to the effective modulus in the transverse direction.

2.3. Porosity

Porosity is defined as the ratio of the void space to the total volume of a solid. Total volume was calculated by measuring length, width and height of rectangular scaffold samples [26]. The mass of the scaffold samples was then used in conjunction with the specific gravity of Tecoflex SG80 polyurethane (1.04 g/cc) to determine the volume of polyurethane in the sample. Void space was the difference in volume between the total volume, and the volume of the polyurethane. Porosity was calculated as the ratio of void space to total volume of the scaffold.

2.4. Cell culture and seeding

Mouse embryo fibroblast NIH-3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FBS and 1% L-glutamine, and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA). All culture media mentioned herein is the same as described above unless otherwise noted. Cells were cultured in a T- flask (Fisher Scientific, Waltham, MA) and media was changed twice per week until they reached approximately 90% confluence. Cells were then passaged. Briefly, culture media was removed from the cells. Cells were then rinsed with sterile PBS to remove any remaining culture media. Trypsin was added to the flask, and allowed to act for 2–4 min. at 37° C. Fresh media was then added to quench the trypsin. Cells were centrifuged and the supernatant discarded. The cell containing pellet was then resuspended in fresh media and transferred to a new T-flask. Cells were passaged three times before seeding onto the scaffold.

Prior to seeding, scaffolds were sterilized by spraying with 70% EtOH and rinsing with sterile PBS and culture media. Cells were removed from the surface of the cell culture flask via trypsinization as described above. These cells were then separated from trypsin through centrifugation and resuspended in cell culture media. Approximately 3.5×10^7 cells were seeded by gently pipetting 500 μl of the cell suspension onto the surface of the scaffold in a 100 mm plastic culture dish (Fisher Scientific). Cells were allowed to attach for two hours according to previously published methods (7) before the scaffold was covered with fresh culture media. Media was replaced with fresh media twice each week for 14 days.

2.5. Cell imaging and analysis

Following cell culture, cells were imaged with fluorescein diacetate (FDA) (Invitrogen) to ensure that viable cells were present. The cells were then fixed with 4% paraformaldehyde, and stained with DAPI (Invitrogen) for nuclei visualization and phalloidin conjugated with alexafluor 488 (Invitrogen) for actin filament visualization according to the manufacture's guidelines. Images were then collected using an Olympus FV 1000 (Center Valley, PA) confocal microscope starting at the surface of the scaffolds, and capturing image slices every 5 μm to a depth of 50 μm. These stacks were then projected into a single image of the maximum intensity pixels through scaffold using ImageJ, (NIH imaging software, Bethesda MD).

Image analysis was performed on the projected images using custom written MATLAB code. A 2D FFT method similar to what is reported by Ayers et al. was used to measure the direction fiber alignment [27]. Briefly, randomly selected regions of cell growth were processed with a Gaussian filter in order to reduce edge effects. A 2D FFT was performed and filtered to include only frequencies from 20–50 pixels. This range was selected based on the average spacing of actin filaments in the images in order to decrease artifacts from larger structures such as scaffold structure. Average pixel intensity was measured at every angle in the frequency range mentioned above. Average pixel intensity was plotted with respect to the angle from horizontal, and shifted 90° to align the peak with the direction of actin filament alignment. Cellular alignment is reported as the orientation index, which is defined as the angle that captures 50% of the actin filament alignment, as determined by the area under the average pixel intensity curve.

2.6. Scaffold lamination

In order to generate thicker materials, scaffolds were laminated during the drying process. Scaffolds were fabricated as described previously and elongated 70%. Instead of drying overnight individually, five scaffolds were placed one on top of another and allowed to dry. During the drying process the scaffolds formed a continuously adhered lamination. After the laminated scaffolds had dried, they were frozen in liquid nitrogen and broken to expose the cross section. The cross section of these thick scaffolds was observed through electron microscopy to qualitatively assess pore structure and the lamination interface.