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Development and characterization of a porous micro-patterned scaffold for vascular tissue engineering applications

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Abstract

The fabrication of functional small diameter blood vessel analogs has implications in vascular disease treatment. Current 3D models of the medial vessel layer lack micron-scale topographical cues that have shown promise in vitro by recapitulating native vascular smooth muscle cell (VSMC) behavior. A major obstacle to fabricating 3D scaffolds is maintaining adequate nutrient diffusion to cells. We have developed and characterized porous micro-patterned poly-caprolactone (PCL) scaffolds using a novel technique that integrates soft lithography, melt molding and particulate leaching of polylactic-*co*-glycolic acid (PLGA) micro/nanoparticles. Scanning electron microscopy showed that PLGA-leached scaffolds have circular pores significantly smaller than the size scale of the grooved surface pattern (48 μm grooves; 5 μm deep; 12 μm spacing). Diffusion of media through PLGA-leached scaffolds was six-fold greater than through non-porous scaffolds, indicating successful introduction of through-pores into PCL by the PLGA leaching technique. VSMC alignment on micro-patterned PLGA-leached scaffolds was similar to that on micro-patterned non-porous scaffolds, indicating no loss in cellular organization on PLGA-leached scaffolds. In contrast, cells seeded on micro-patterned sodium bicarbonate-leached scaffolds remained un-aligned. The ability to micro-pattern cells on porous scaffolds may facilitate the transfer of micro-technology from simple 2D substrates to complex 3D architectures, allowing for tight control over cellular organization in fabricated tissues.
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1. Introduction

The prevalence of cardiovascular disease and inherent problems with current surgical therapies has prompted major efforts in the field of vascular tissue engineering. While there have been significant advances towards creating functional blood vessel substitutes, the fabrication of a vascular graft with mechanical, structural and functional properties similar to native vessels has not yet been achieved [1].

In native vessels, the medial layer consists of highly organized layers of vascular smooth muscle cells (VSMCs). The cells in these layers are oriented at different angles in alternating layers creating a herringbone helical arrangement of cells that is thought to promote the strength and stability of the vessel [2]. One approach that has been used to form the medial layer in vitro is the fabrication of cell sheets that can be assembled and matured into a tissue [3]. Although tissues from this 'cell sheet method' remain fully functional 7 days after implantation, drawbacks include long culture times and lack of cellular organization in the vessel wall [1,3]. Incorporation of the in vivo cellular and ECM organization may assist in decreasing vessel culture time by enhancing ECM remodeling [4] as well as in increasing vessel strength and stability by providing VSMCs with appropriate environmental and organizational cues for proper functionality.

Cell patterning using topographical cues in the form of grooves has shown promise in aligning VSMCs, eliciting in vivo like VSMC morphology and promoting increased ECM remodeling [4,5]. Studies have further shown, that with increasing pattern size, control over VSMC behaviors such as alignment and ECM remodeling are lost [4].

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The goal of this study was to develop a 2D biodegradable vascular graft scaffold with micron-scale features as a basis for creating 3D tissue structures. The biopolymer poly-caprolactone (PCL) was chosen because of its ease of processing (melting temperature 58–63 °C [6]), biocompatibility [7] and the ability to manipulate its mechanical and degradation properties by the formation of PCL co-polymers [8,9]. When fabricated through melting techniques, PCL as well as other dense polymers, generally lacks any voids and pores. This lack of pores limits gas and nutrient exchange [10]. Adequate nutrient diffusion through the scaffold layers however is essential for proper vessel functionality and viability, i.e. the scaffold should not only promote cellular organization but also allow nutrients to pass through to underlying layers. Thus the scaffold must incorporate an interconnected porous network that does not interfere with cellular organization.

Currently, no technique is available to fabricate such structures [11]. Strategies to introduce porous architectures into 3D scaffolds include particulate leaching of salts and sugars [12] phase separation and freeze drying [13]. These techniques, however, produce relatively large pores on the order of tens of microns or larger. At these size scales, cells conform to the porous micro-architecture presented [14] which, especially in the case of highly organized tissues such as blood vessels, does not provide the precise control needed to organize cells into the highly aligned structures found in vivo. In this study we developed a novel scaffold fabrication method for the incorporation of a porous network in micro-patterned dense polymer scaffolds to improve nutrient diffusion while simultaneously limiting disruption of surface micro-patterning.

2. Materials and methods

2.1. Fabrication and quantification of polylactic-co-glycolic acid (PLGA) micro/nanospheres

In order to create micro-patterned biodegradable scaffolds with non-disruptive pores, PLGA micro/nanospheres were fabricated for use as a porogen. Microspheres containing Texas red (TR-DHPE (Sigma Aldrich, St. Louis, MO)) (used for imaging purposes) were formulated using a double emulsion-solvent evaporation technique modified from Panyam et al. [15]. TR-DHPE at a volume of 30 μL, was added to a glass cuvette and dried using argon gas. Medisorb 5050 DL PLGA (150 mg (Alkermes, Cambridge, MA)) and methylene chloride (2.5 mL (Sigma Aldrich, St. Louis, MO)) were added to the dried TR-DHPE. The solution was then emulsified using a sonicator for 2 min (Aquasonic, Model 75 T). The water-in-oil emulsion formed was further emulsified into an aqueous solution of poly(vinyl alcohol), (PVA) (35 ml, 0.5% w/v (Sigma Aldrich, St. Louis, MO)) using a homogenizer (High Shear Lab Mixer, L4RT-Ai Silverson) for 5 min to form multiple water-in-oil emulsions. The multiple emulsions were then stirred for approximately 18 h at room temperature in the dark. The PLGA microspheres were then centrifuged (Eppendorf 5804), washed two times to remove PVA and unentrapped TR-DHPE, and then lyophilized in a Benchtop Lyophilizer (Virtis, Gardiner, NY) for 48 h to obtain the micro/nanospheres in dry powder form. To achieve greater yield of micro/nanospheres PLGA and PVA concentrations were tripled, in the micro/nanospheres fabrication process to fabricate the desired amount of spheres.

2.2. Fabrication of porous patterned PCL thin film scaffolds

2.2.1. Fabrication of thin micro-patterned PCL scaffolds

A mechanical heating press was used to fabricate micro-patterned PCL scaffolds with thicknesses on the tens of microns scale. To create a thin film micro-patterned tissue scaffold, a patterned poly-dimethylsiloxane (PDMS) (Dow Corning, Midland, MI) mold fabricated using conventional micro-fabrication and soft lithographic techniques [4] was used to transfer topographical features (48 μm grooves spaced 12 μm apart and 5 μm deep) into PCL. The textured side of the PDMS mold was placed onto a thin 1 g block of PCL (MW 43,000–50,000 (Polyscience Inc., Warrington, PA)). The PCL/PDMS was then placed into a custom-designed mechanical heating press and the PCL was melted at 110 ± 10 °C for 5 min. A pressure of approximately 9.4 psi for pure PCL, 9 psi for PLGA embedded PCL or 28 psi for sodium bicarbonate (SB)-embedded PCL was applied to the PCL/PDMS for 5 min to force molten PCL into the PDMS channels and produce a scaffold with even thickness of approximately 50 μm. The press was then rapidly cooled with a water flow system to room temperature for 5 min. With parameters held constant (i.e. temperature, PCL dimension and weight, composition and compression time) the thickness of the PCL thin film could be controlled by the variation in pressure.

2.2.2. Fabrication of porous PCL scaffolds using PLGA micro/nanospheres and SB particles

In order to introduce pores in thin micro-patterned PCL scaffolds, both a traditional and a novel porous fabrication technique were investigated. The conventional technique of using salt leaching has been investigated here as a means of comparison to the leaching of PLGA micro/nanospheres for the introduction of sub-micron scale pores.

SB (Sigma Alrich, St. Louis, MO) or PLGA micro/nanospheres were incorporated into molten PCL at weight ratios of 2:1 and 4:1 PCL:SB, and 2:1 and 4:1 PCL:PLGA, respectively, to form thin blocks of the composites. The much higher melting temperature of PLGA enabled the PCL to be in a molten state while leaving the PLGA micro/nanospheres intact. These composite blocks were then used in the mechanical heat press as described earlier, resulting in thin micro-patterned PCL scaffolds with embedded SB or PLGA particles. SB-incorporated thin film scaffold were immersed in warm distilled water (40 °C) and shaken gently in a warmed shaker bath (New Brunswick Scientific, Classic Series C78). Water was changed every 2 h for 10 h and then 2–3 times daily for 4 days to leach out SB particles. This leaching technique and time frame has been shown previously to adequately leach accessible salt particles [13]. PLGA-incorporated thin film scaffolds were immersed in a 0.5 M solution of sodium hydroxide, NaOH (Sigma Aldrich, St. Louis, MO) and shaken gently for 30 min on a shaker plate to rapidly degrade the micro/nanospheres. Since PLGA degrades at a much faster rate than PCL [6], the PCL micro-structure is not affected by the PLGA micro/nanosphere leaching process. After the NaOH soak, scaffolds were rinsed in distilled water until the rinsing solution reached a neutral pH as demonstrated by pH strips.

2.3. Evaluation of scaffold structure

PLGA micro/nanosphere proximity to each other and distribution throughout the PCL scaffold was investigated using a confocal laser microscope (CLM, Olympus Fluoview version 2.1 with Tiempo mounted on an upright Olympus BX50 microscope) equipped with a krypton laser (568 nm). *Z*-stack images of the scaffold were obtained every 1 μm through the thickness of the scaffold and rendered using Olympus Fluoview data acquisition software and Image J. To evaluate scaffold structure after SB and PLGA leaching, scanning electron microscopy (SEM) (JSM-6100, Peabody, CA) was used to image scaffold surfaces and cross-sections. Scaffolds were coated with a 20-nm-thick gold coating and images were taken at 5 kV.

2.4. Evaluation of nutrient diffusion through tissue scaffolds

A diffusion set-up previously described by Leoni et al. [16] was used to evaluate nutrient diffusion through the fabricated PCL scaffolds.

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