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The influence of stereolithographic scaffold architecture and composition on osteogenic signal expression with rat bone marrow stromal cells

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ABSTRACT

Scaffold design parameters, especially physical construction factors such as mechanical stiffness of substrate materials, pore size of 3D porous scaffolds, and channel geometry, are known to influence the osteogenic signal expression and subsequent differentiation of a transplanted cell population. In this study of photocrosslinked poly(propylene fumarate) (PPF) and diethyl fumarate (DEF) scaffolds, the effect of DEF incorporation ratio and pore size on the osteogenic signal expression of rat bone marrow stromal cells (BMSCs) was investigated. Results demonstrated that DEF concentrations and pore sizes that led to increased scaffold mechanical stiffness also upregulated osteogenic signal expression, including bone morphogenic protein-2 (BMP-2), fibroblast growth factors-2 (FGF-2), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and Runx2 transcriptional factor. Similar scaffold fabrication parameters supported rapid BMSC osteoblastic differentiation, as demonstrated by increased alkaline phosphatase (ALP) and osteocalcin expression. When scaffolds with random architecture, fabricated by porogen leaching, were compared to those with controlled architecture, fabricated by stereolithography (SLA), results showed that SLA scaffolds with the highly permeable and porous channels also have significantly higher expression of FGF-2, TGF-β1, and VEGF. Subsequent ALP expression and osteopontin secretion were also significantly increased in SLA scaffolds. Based upon these results, we conclude that scaffold properties provided by additive manufacturing techniques such as SLA fabrication, particularly increased mechanical stiffness and high permeability, may stimulate dramatic BMSC responses that promote rapid bone tissue regeneration.

1. Introduction

Scaffold design parameters are considered important in order to achieve a functional complex of cell/scaffold constructs, including pore size, porosity, interconnectivity, surface properties, mechanical strength, the amounts and types of filler material, cell seeding density, and exogenous growth factors [1]. In general, transplanted cell population may recognize the differences in these physical and mechanical cues and the subsequent cellular functions might be changed. Modulation of scaffold physical properties as well as changes in scaffold design parameters may influence the various cellular functions. Both stiffness (mechanical cues) and pore geometry (architectural cues) among these parameters are of importance to upregulate the endogenous osteogenic signal expression.

It is known that the scaffold stiffness influences adhesion [2], motility [3,4], morphology [5–7], proliferation [3,8,9], and osteoblastic differentiation [8–13] of cells. Cells can recognize scaffold mechanical cues (e.g., stiffness) and respond with secondary signal transduction that can bring about cell–matrix interaction [14]. Recent studies have revealed that specific lineage of stem cell differentiation cascades can be directed by matrix elasticity [13,14]. In particular, it has been shown that mechanical properties of extracellular matrix (ECM) may regulate the osteogenic signaling mechanisms of cell–ECM through the sequential activation of FAK, RhoA/ROCK, MAPK, and Runx2 [3,11,12]. This osteogenic mechanotransduction can be also enhanced by a combination of mechanical cues with other stimuli such as ligand presentations on ECM [7].

In addition to mechanical stimuli, architectural cues for a 3D porous scaffolds including porosity, pore size, interconnectivity, and channel orientation are also important design parameters that can affect osteogenic signal expression of a seeded cell population

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[1,15]. A study with human mesenchymal stem cells on coralline hydroxyapatite scaffolds has shown that pore size could be a factor controlling both the level of bone morphogenetic protein-2 (BMP-2) mRNA expression and osteoblastic differentiation [16]. Similarly, another *in vitro* study also demonstrated that porous architecture of 3D silk fibroin scaffolds with the optimized porosity facilitated an increase in osteoblastic phenotypes of BMSCs [17]. An *in vivo* study with β-tricalcium phosphate scaffolds has shown that significantly higher osteoblastic differentiation was observed in higher porosity (over 65%) groups than in lower porosity groups [18]. Moreover, continuous pore geometry in scaffolds manufactured via solid freeform fabrication (SFF) has shown a higher cell ingrowth depth compared to scaffolds with random pore architecture [19].

In order to investigate the effect of mechanical and architectural cues on the stimulation of osteogenic signal expression, a composite material of poly(propylene fumarate) (PPF) and diethyl fumarate (DEF) was used in this study. This composite has shown unique photo-crosslinking characteristics [20]. By changing the molecular weight of PPF, the amount of photoinitiator and the ratio of PPF/DEF, the crosslinking density and mechanical properties of the PPF/DEF composite can be modulated. Due to this controllability, the mechanical stiffness of PPF/DEF scaffold can easily modulated during fabrication process. Besides of the controllable stiffness, PPF/DEF is a useful resin material for stereolithography (SLA). Incorporation of DEF with PPF reduces the viscosity of this liquidic polymeric mixture making it easier to utilize for SLA. The SLA device uses a laser to initiate the resin photo-crosslinking reaction and fabricate a 3D scaffold by vertical layering. SLA is one of the most versatile SFF techniques due to its accuracy, precision, and computer aided pre-design of the 3D external and internal scaffold geometry. SLA has been found useful for the rendering of patient- and defect-specific bone implants based on a patient's 3D CT scan [21,22]. SLA can also control the scaffold design parameters such as pore architecture and mechanical stiffness by modulating of photo-crosslinking reaction.

The global hypothesis of this study is that the modification in design parameters of 3D PPF/DEF composite scaffolds may facilitate osteogenic signal expression and enhanced level of signal expressions associated with the downstream osteoblastic differentiation of a seeded cell population. Therefore, the first part of this study is an investigation of the effect of DEF contents and pore size on the endogenous osteogenic signal expression and downstream osteoblastic differentiation of seeded bone marrow stromal cells (BMSCs) on 3D PPF/DEF scaffolds. It should be emphasized that changing the DEF incorporation ratio in PPF/DEF composite scaffold alters the crosslinking density and mechanical stiffness of the resulting scaffold. This sequential modulation in properties of the scaffold could stimulate the upregulation of osteogenic signal expression. The second part of this study is to investigate the effect of pore geometry within a scaffold, as another architectural cue, on the early osteogenic signaling profiles. For this second object, the advantage of controlled channel geometry of 3D macroporous PPF/DEF scaffolds, fabricated by SLA, over random pore structure, fabricated by porogen leaching method, on the osteogenic signal expressions has been investigated for the first time.

The specific objective of this study are: (1) to characterize the physical properties of 3D macroporous PPF/DEF composite scaffolds, (2) to investigate the effect of DEF content (subsequent changes in stiffness as a mechanical cue) and pore size (architectural cue) on osteogenic signal expression profiles and downstream osteoblastic differentiation, and (3) to investigate the effect of pore geometry (i.e., continuous channel geometry versus random pore structure) on the osteogenic signal expression of rat BMSCs.

2. Materials and methods

2.1. PPF synthesis and scaffold fabrication

PPF was synthesized according to previously reported methods [23]. Briefly, DEF and propylene glycol were reacted with zinc chloride as a catalyst and hydroquinone as a crosslinking inhibitor to form an intermediate compound. Then, transesterification was occurred to create the final PPF under vacuum condition. The number average molecular weight of the purified PPF (M_n = 1200 Da in this study) was determined by gel permeation chromatography. For PPF/DEF composite scaffold fabrication, PPF was mixed with DEF with various weight ratios (Table 1). 75 wt% of salt porogen crystals (>500 μm for "large" pore size and 180–300 μm for "small" pore size) and 0.5 wt% of photoinitiator bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO, Ciba Specialty Chemicals, Tarrytown, NY) were then homogeneously mixed with the PPF/DEF polymer mixture. The resulting paste was packed into a glass cylinder mold and photocrosslinked under UV light (intensity of 2.68 mW/cm^2) for 2 h. Crosslinked polymer networks with salt porogens were cut into disks (6 mm in diameter and 3 mm in thickness) and placed in water for 3 days to leach out salt. The resulting macroporous PPF/DEF scaffolds were air-dried for 24 h and then dried again in a vacuum for 24 h. All experimental groups with random pore structure are listed in Table 1.

2.2. SEM imaging

The top surface of scaffolds was visualized using a scanning electron microscope (SEM) (SU-70, Hitachi, Tokyo, Japan). Samples were gold sputter-coated. The images were obtained at 3 kV accelerating voltage.

2.3. Sol fraction

To assess the crosslinking density of PPF/DEF scaffolds, sol fraction test was performed by a previous method [20]. Each photocrosslinked scaffold was placed in 20 ml of methylene chloride solvent in a glass vial. The weight of the initial sample before incubation in solvent (W_i) was measured, and the samples were then incubated on a shaker at 75 rpm for 160 h at room temperature. Then, samples with solvent were transferred onto a weighed filter paper (W_p). These were completely dried in an oven at 70 °C for 2 h and weighed again (W_{p+s}). The sol fraction was calculated from the formula: sol fraction = $(W_i - (W_{p+s} - W_p))/W_i$. Five independent samples were assessed (n = 5).

2.4. Mechanical properties

According to the American Society of Testing Materials (ASTM) Standard D695-2a, compressive mechanical testing was performed using an Instron (Norwood, MA) mechanical tester (Instron 5565) to measure the compressive modulus and offset yield strength. The cylindrical porous scaffolds with 6 mm in diameter and 12 mm in length were compressed along its vertical axis. Compression was applied at a speed of 1.3 mm/min until the compressive strain reached 0.5 mm/mm. The compressive modulus and yield strength at 1% offset were calculated using Bluehill 2.16 software (Instron). Four replicates in each experimental group were tested (n = 4).

2.5. Permeability

The water permeability of scaffolds was determined according to the methods previously described based on Darcy's law [24,25]. An apparatus was constructed using a 2-L open container functioning as a water reservoir large enough to keep the pressure across the scaffold nearly constant (i.e., by keeping the height of the water in the apparatus nearly constant). Attached to the bottom of this reservoir was a short tube in which the scaffold, first wrapped in parafilm to create an air-tight seal along the side wall, was held. 1 L of water was added to the reservoir, and the water penetrated through the scaffold vertically was collected. After 120 s, the mass of water collected was recorded and the mass flow rate was calculated. This mass flow

Table 1
Experimental groups with random pore architecture: L = "large" pore (i.e., >500 μm), S = "small" pore (i.e., 180–300 μm).

Experimental groups	PPF	DEF	Pore size (μm)	Leached porogen amount (wt%)
L1	100	–	>500	77.41 ± 0.48
L2	90	10	>500	79.51 ± 1.14
L3	75	25	>500	78.43 ± 0.55
L4	66	33	>500	81.67 ± 0.58
S1	100	–	180–300	75.36 ± 0.24
S2	90	10	180–300	77.37 ± 0.52
S3	75	25	180–300	79.77 ± 0.58
S4	66	33	180–300	77.48 ± 0.37