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Osteogenic cell functionality on 3-dimensional nano-scaffolds with varying stiffness

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ABSTRACT

Creating implants that lead to optimal bone remodeling has been a challenge for more than two decades because of a lack of thorough knowledge of cell behavior in three-dimensional (3D) environments Limitations in traditional fabrication techniques and difficulties in characterizing cell-scaffold interactions have limited our understanding of how factors like scaffold pore size and distribution, as well as stiffness affect cell response

To date, cellular activity on 3D substrates with stiffness ranging from a few kPa to hundreds of MPa has been investigated extensively (Cui et al., 2009; Fu et al., 2011; Hulbert et al., 1970; Hollinger et al., 1996; Johnson and Herschler, 2011; Karageorgiou and Kaplan, 2005). Fabrication limitations have restricted scaffolds with strut dimensions on the order of a few microns, a size comparable to the dimensions of osteoblasts, to have compressive moduli ranging from 10 kPa to 200 kPa, which has limited our understanding of how scaffolds stiffness affects mineral deposition. Cell viability and functionality on 3D scaffolds with compressive moduli in the MPa range and with strut dimensions on the order of a few microns have not yet been reported. We employed two-photon lithography to create periodic 3D nano-architectures with ∼99% porosity, ∼ 2 μm strut diameters, and ∼2–9 MPa structural stiffness to explore the influence of scaffold properties on the viability of osteoblasts in a microenvironment similar to that of natural bone. These nanolattices were made out of a polymeric core coated with different materials and had unit cells with tetrakaidecahedral geometry and a 25 μm pore size. The unit cells were tessellated in space to form a lattice with lateral dimensions of 200 × 200 μm and a height of 50 μm. Some of the polymer nanolattices were coated with a conformal 120 nm-thick layer of SiO_2, others were coated with 120 nm of Ti. All nanolattices had a ∼20 nm-thick outermost layer of TiO_2. Osteogenic cells were grown on the nanolattices for 28 days and the resulting cell morphology and depositions were characterized via scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), and Raman spectroscopy. These analyses revealed significant cell attachment and the presence of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), tricalcium phosphate ($Ca_3(PO_4)_2$) and metaphosphates ($[Ca_2(P_2O_7)]_n$), chemical species normally found in natural bone. Such osteogenic functionality suggests that 3-dimensional nano-architected materials can be used as effective scaffolds for cell growth and proliferation, which could eventually lead to the generation of better bone implants.

1. Introduction

Bone grafting is among the most common surgeries in the US with approximately 400,000 cases per year [1]. Titanium alloys represent the most widely adopted materials for use in bone implants because of their excellent biocompatibility, high strength, fracture toughness, and reliable mechanical performance as replacement for hard tissues.

One problem that has been identified in these materials is the mismatch between the elastic modulus of the implant, on the order of hundreds of GPa [2], and that of bone, which ranges from 0.2 to 10 GPa for trabecular bone and from 22 to 26 GPa for cortical bone [3,4]. This mismatch in moduli leads to stress shielding, a phenomenon by which the orthopedic implant absorbs most of the imposed mechanical load and minimizes load transfer to the surrounding cells [5].

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Wolff's law predicts two possible outcomes for bone growth in response to mechanical loading: (1) applying an adequate mechanical load to bone causes the surrounding osteoblasts to respond by initiating a remodeling process that leads to the formation of denser and stronger bone over time or (2) a lack of mechanical load on bone activates osteoclasts, which start breaking down bone tissue which leads to bone resorption [6]. Clinical data confirms these predictions and highlights the importance of load transfer to bone cells [7]. When bone fracture occurs, the load transfer is interrupted, and an implanted orthopedic device takes on the function of re-establishing structural support and load transfer.

Several studies have demonstrated that the relatively higher modulus of titanium-based implants with respect to natural bone causes little to virtually no load transfer from the implant to the surrounding tissues, which leads to bone resorption and to the generation of weaker bone, which subsequently increases the chances of fracture recurrence [2,5,6].

In addition to modulus matching, an effective implant has to provide an ideal microenvironment for osteoconduction and osteoinduction that facilitate osteogenesis. Existing literature has largely focused on investigating cell behavior on 2-dimensional substrates (2D), whose properties like stiffness and surface roughness are readily obtained [8–12]. The parameter space in 3-D is more complex and involves investigating the effects of relative density, effective surface area and scaffold's structural stiffness on cell function. The latter is usually correlated with the deposition of collagen and calcium phosphate.

Porous 3-D scaffolds offer a useful platform to investigate key parameters of bone remodeling for the eventual design of more effective implantable orthopedic devices [13]. One example of such scaffolds is rigid cellular solids, which are assemblies of geometrical unit cells that pack together to fill space and are commonly found in nature: the alveolar micro-architecture of the lung and the trabecular bone network are examples of cellular architectures found in the human body.

A key descriptive feature of cellular solids is their relative density ($\bar{\rho}$), which is defined as the volume fraction of the solid material (V_s) divided by the representative volume of the unit cell (V_{uc}) [14,15].

$$\bar{\rho} = \frac{V_s}{V_{UC}}. \tag{1}$$

Relative density is a function of unit cell topology, mean pore size and the ratio of strut length to strut cross sectional area (L/a^*b) as shown in Fig. 1(A). The structural modulus of elasticity, E^*, for a periodic cellular solid, scales with the relative density, ($\bar{\rho}$), in a power law fashion as:

$$E^* = CE_s(\bar{\rho})^m \tag{2}$$

where C is a geometry-dependent proportionality constant and E_s is the elastic modulus of the solid that comprises the unit cell [16,17,15]. Another key parameter of cellular solids is specific surface area, SSA, which is also a function of relative density and is defined as:

$$SSA = \frac{SA}{V} = \frac{3.65}{L}(\bar{\rho})^{0.5} \tag{3}$$

where SA is the surface area available to the cells to attach, V is the total volume occupied by the unit cell and L is the length of the unit cell strut [18]. This implies that varying relative density of cellular solids by, for example, changing the dimensions and/or the geometry of the unit cell, offers a high degree of control and tunability of their modulus and specific surface area.

Recent research has shown that mammalian cell viability, attachment and migration strongly depend on mean pore size and specific surface area of the 3D cellular scaffolds [16,19–22]. O'Brien et al. discovered that as the pore size increased from 95 to 150 μm, cell viability decreased by a factor of 2 and cell attachment scaled linearly with increasing specific surface area [23]. Harley et al. showed that cell migration and cell speed, measured as the distance covered by a cell in a given amount of time increased by a factor of 2 when the pore size was reduced from 151 to 96 μm [17]. The described works have either employed 3D scaffolds with MPa-level structural stiffness and strut dimensions of hundreds of microns, which is an order of magnitude larger than the osteoblast cell size, or strut dimensions on the same order as cell size (1–10 μm), whose stiffness spans 10–200 kPa [24–29,20,30,31]. Using two-photon lithography in this work allowed us to investigate cell viability and functionality on cellular solids with strut dimensions on the same order as the osteoblast cell size and with structural stiffness reaching into the MPa region.

2. Materials and method

2.1. Sample preparation

We used direct laser writing (DWL) two-photon lithography to first fabricate 3-dimensional periodic nanolattices of interconnected polymer beams, with a specific surface area of 0.061 μm^{-1} and a relative density of 1.23%, close to that of trabecular bone, 5%. We chose a tetrakaidecahedral unit cell (Fig. 1(B)) to mimic the porous structure of trabecular bone, which responds similarly to applied mechanical loading. The pore size in these nanolattices was $U = 25$ μm, measured as the linear distance from one square face to the opposite one, the beam length (L) was 8.33 μm and the beams had an elliptical cross section with a major axis ($2a$) of 2.24 μm and a minor axis ($2b$) of 1.3 μm (Fig. 1(A)). Each nanolattice contained $8 \times 8 \times 2$ unit cells (Fig. 1(C)) and each sample contained 4 nanolattices arranged in a square (Fig. 1(D)). Nanolattices were made of three material systems: (i) Polymer nanolattice IP-Dip (Nanoscribe GmbH) coated with a 20 nm thick layer of TiO_2 deposited via Atomic Layer Deposition (ALD); (ii) polymer nanolattice coated with 120 nm of sputtered SiO_2 and the same outermost ALD coating of 20 nm TiO_2 as (i); and (iii) polymer nanolattice coated with 120 nm of sputtered Ti and the same outermost ALD coating of 20 nm TiO_2 as (i) and (ii) (Fig. 1(A)).Sputter deposition was carried out using a TES magnetron sputterer. Titanium was sputtered using RF power at 125 W, a working pressure of 10 mtorr, Ar pressure of 100 sccm and table rotation set at 100%. An average Ti thickness of 120 nm was obtained after depositing for 70 min. SiO_2 was deposited using RF power of 125 W, a working pressure of 10 mtorr, Ar and O_2 as sputtering gases with a relative concentration of 80%–20% and table rotation set at 100%. The deposition occurred over 180 min to obtain an average coating of 120 nm. All 3 material systems were coated with a 20 nm-thick outermost layer of TiO_2 that was deposited using a Cambridge Nanotech S200 atomic layer deposition (ALD) system with H_2O and Titanium Tetrachloride ($TiCl_4$) as precursors. The relative density of the nanolattices was calculated using Solidworks software by evaluating the volume fraction of the solid material and dividing it by the representative volume of the unit cell and specific surface area was calculated using Eq. (3).

2.2. Beam stiffness calculations

We performed analytical calculations to estimate the stiffness of individual struts that comprise the nanolattice. Strut stiffnesses were used to evaluate whether the seeded cells would be able to cause any significant bending of the struts. The slenderness ratio (L/a) of the beams is ∼10, which is in the regime of applicability of the Euler–Bernoulli beam bending theory. It is reasonable to assume that the seeded cells exert a distributed load, q, of