A synergistic approach to the design, fabrication and evaluation of 3D printed micro and nano featured scaffolds for vascularized bone tissue repair

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ABSTRACT

3D bioprinting has begun to show great promise in advancing the development of functional tissue/organ replacements. However, to realize the true potential of 3D bioprinted tissues for clinical use requires the fabrication of an interconnected and effective vascular network. Solving this challenge is critical, as human tissue relies on an adequate network of blood vessels to transport oxygen, nutrients, other chemicals, biological factors and waste, in and out of the tissue. Here, we have successfully designed and printed a series of novel 3D bone scaffolds with both bone formation supporting structures and highly interconnected 3D microvascular mimicking channels, for efficient and enhanced osteogenic bone regeneration as well as vascular cell growth. Using a chemical functionalization process, we have conjugated our samples with nano hydroxyapatite (nHA), for the creation of novel micro and nano featured devices for vascularized bone growth. We evaluated our scaffolds with mechanical testing, hydrodynamic measurements and *in vitro* human mesenchymal stem cell (hMSC) adhesion (4 hours), proliferation (1, 3 and 5 days) and osteogenic differentiation (1, 2 and 3 weeks). These tests confirmed bone-like physical properties and vascular-like flow profiles, as well as demonstrated enhanced hMSC adhesion, proliferation and osteogenic differentiation. Additional *in vitro* experiments with human umbilical vein endothelial cells (HUVEC) also demonstrated improved vascular cell growth, migration and organization on micro-nano featured scaffolds.

Keywords: 3D printing, tissue engineering, annomaterials, osteogenesis, neovascularization

1 INTRODUCTION

Today in the field nanostructured materials have already been popular for growth of blood vessels and biomimetic vascular networks to further enhance tissue growth. Specifically, bone has been targeted as a model system for some of these studies. Here we have combined nanomaterials and 3D printing for a highly innovative complex 3D printed scaffold with both nano and micro features for both bone and vascular growth. Key innovations of this project include the design and fabrication of a fully interconnected 3D fluid perfusable microchannel network, within a microstructured bone forming matrix. Also in this study we designed and achieved a unique integration of nanocrystalline hydroxyapatite (nHA) into our 3D printed scaffolds using a post fabrication process. We incorporated hydrodynamic measurement of unsteady pressure and flow rates. These measurements facilitate a preliminary understanding of the causal effects of predesigned structure-induced flow perturbations and the efficacy of such structures. Our motivation to study arterial blood flow in context of predesigned vascular structures is due to the essential role of blood supply for the growth of large critical-sized bone tissue. We envisioned that vascular network in the 3D printed scaffolds would experience flow conditions with some intrinsic vascular flow features such as flow rates, pressures and pulsatility. We therefore, modeled the hydrodynamic experiments under similar flow conditions in an exclusive arterial flow loop, recreating those salient cardiovascular flow characteristics. Ultimately, we believe that vascular flow properties and pulsatility may have a greater role to play toward fast, *in situ* delivery of blood, nutrients, progenitors and growth factors through our predesigned vascular structures. Cellular study was also conducted to prove scaffolds' effectiveness in enhancing cell growth and tissue formation, and physical characterization was performed to show desirable, bone like characteristics.

2 METHODS

Scaffolds' outer shape was a 7.5 mm diameter and a 5 mm high cylinder. For the bone region, the scaffolds had a 250 µm diameter hexagonal pore size and a 375 µm layer height. The pores in the bone regions were closely packed and then stacked layer by layer, as opposed to the fluid flow

microchannel networks which were long interconnected channels. In order to further dampen fluid perfusion through the bone matrix, and provide a more biomimetic network for microvascular vasculature to develop on, we sought to provide a disrupted, yet highly directionalized flow path perpendicular to the vertical alignment of the hexagonal pores through the scaffolds' pore structures by alternating the scaffold geometry between a simple line pattern and a hexagonal pore pattern. That is to say, the first layer of scaffold pore structure was an arrangement of interlocking hexagons in the X-Y plane, while moving up another full layer in the Z-direction the pore structure changes to a simple series of aligned struts. This pattern then alternates between hexagons and struts with each layer

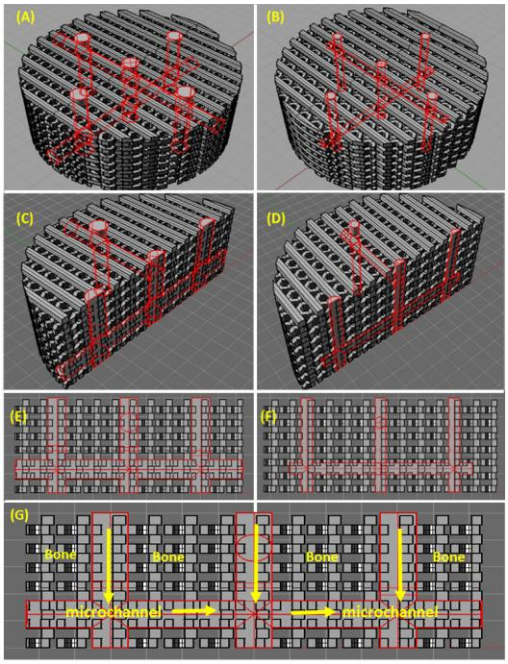

Figure 2 3D CAD modeling of scaffolds with (A, C, E) 500 µm microchannels and (B, D, F) 250 µm microchannels. (G) illustrates the flow patterns in the microchannels of the designed scaffolds.

(**Figure 1)**.

After initial 3D fabrication, scaffolds were additionally modified with a nHA conjugation. nHA was first synthesized using a wet chemistry procedure and a hydrothermal process, as thoroughly described in our previous studies [1, 2]. Then, nHA particles were conjugated onto scaffolds using a process described by Aishwarya et al [3] and our previous study [4]. First, PLA scaffolds were aminolysed. This was achieved by immersing them in an ethylenediamine/n-propanol (1:9 ratio) solution at 60 ºC for 5 min. Scaffolds were then extensively washed with deionized water and dried at 35 ºC. This results in the presence of an NH_2 amine group on the PLA substrate. The aminolysed scaffolds were then immersed in a 1% gluteraldehyde solution at room temperature for 3 h to conjugate CHO functional groups onto the NH_2 groups. After washing extensively, scaffolds were immersed in a solution of 10% w/v nHA at 4 ºC for 24 h. The process itself yields a series of layered chemical attachments, which includes an NH_2 amino group attached to the PLA, CHO functional groups formed off of the amines and finally nHA conjugated on the CHO functional groups.

5 scaffolds from both microchannel groups were tested. Scaffolds were tested using a Lucas Scientific FLS-I portable mechanical tester. A 3 cm compression platen was fitted to the advancing end of the piston, and scaffolds were compressed at a strain rate of 0.1 cm/minute, until failure. Data was collected and analyzed in Excel. The slope of the linear elastic region of each sample's produced stress strain graph was calculated in order to find the Young's Modulus. Samples were imaged using a Zeiss SigmaVP scanning electron microscope (SEM). Scaffolds were coated with a roughly 10 nm thick conductive gold layer using a gold sputter coater. Scaffolds were then imaged using 3.65 kV electron beam. Additionally, scaffold which were not coated with gold were mounted on carbon tape and analyzed using an Oxford Instruments Energy Dispersive X-ray (EDX) spectroscope , in order to characterize the elemental composition present on scaffolds' surfaces.

Hydrodynamic experiments and data collection were performed using a custom made, 180-degree curved artery test section (with curvature ratio, r/R = 1/7) setup designed to represent a dynamically similar pulsatile arterial blood flow through a single arterial vessel [5,6]. The closed loop experimental setup consists of a fluid reservoir, inlet and outlet pipes, a programmable pump and a 180-degree curved tube test section. The inlet and outlet pipes are made of acrylic and approximately, 2 meters long and are connected to a removable 180° curved test section. A Newtonian blood-analog fluid is supplied using a programmable pump (ISMATEC BVP-Z) to tubes containing the scaled model-scaffolds. . The inflow conditions are based on a carotid artery-based digitized flow rate waveform reported in a paper by Holdsworth et al.

hMSCs were obtained from the Texas A&M Health Science Center, Institute for Regenerative Medicine, and were expanded originally from a donor source. Additionally, HUVECs were purchased from Life Technologies. All hMSC studies were cultured in complete cell media (CCM) consisting of alpha minimum essential medium, 16% fetal bovine serum (FBS), 1% L-glutamine, and 10 µg/mL of ciprofloxacin. hMSC osteogenic differentiation studies were cultured in CCM supplemented with 50 µg/mL L-ascorbate acid (Sigma) and 10 mM β-glycerophosphate (Sigma). HUVECs were cultured in endothelial growth media consisting of Medium 200 and 2% low serum growth supplement (LSGS) both purchased from Life Technologies. hMSC and HUVEC *in vitro* studies on our constructs were conducted as follows.

For hMSC and HUVEC adhesion, printed bone scaffolds were seeded with 50,000 cells per scaffold.

Sample groups included scaffolds with large and small microchannels, and scaffolds with large and small microchannels conjugated with nHA. Samples were reacted with a Molecular Probes MTS cell counting reagent and incubated at 37 ºC for one hour. Samples were then read on a Thermo Scientific Multiskan GO photometric plate reader at 490 nm. hMSC and HUVEC proliferation was conducted at 1, 3 and 5 days. Samples were seeded with 55,000 cells per scaffold, cultured in CCM and counted at each time point using the same MTS assay described above. hMSC differentiation studies were seeded with 125,000 cells per scaffold and cultured for three weeks. Samples were tested for calcium and collagen type I deposition. Fluorescent labeling and confocal imaging were also used to further characterize hMSC spreading and morphology on our constructs.

All quantitative material testing and cellular studies were conducted with either a sample size of n=3 or three repeated experiments with total samples size of n=9 per group for each time point, respectively. All quantitative data was compared using a student's t-test, with a p value less than 0.05 taken as "statistically significant."

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3 RESULTS AND DISCUSSION

In this study, a series of well-defined microfeatured bone scaffold were printed, within the well-established resolution for 3D printing. Post fabrication nHA was readily and effectively applied to our scaffolds using a aminolysis based conjugation process. This allowed us to effectively create a highly novel micro and nano featured scaffold for enhanced bone and vascular cell growth. Our scaffolds also performed in compression, comparably to natural bone. These results showed that as the size of blood vessel microchannels was decreased, the Young's Modulus increased. However, this is due to the reduction of void space in the scaffold structure, and this is a well-known structural phenomenon in material science and solid mechanics. All of these results demonstrate very plainly that we were able to design and fabricate scaffolds to highly specified and bone-like characteristics, and these scaffolds then exhibited bone-like physical, particularly mechanical, properties.

As previously discussed, a blood supply is essential for the growth of large critical sized bone tissue. Some researchers in the field have begun utilizing flow perfusion bioreactors, to influence different cell types to form highly structured, micro-vascularized bone. But, these methods rely on extensive *in vitro* or *ex vivo* culture of tissues before they can be theoretically used clinically. Therefore, it is highly clinically relevant to design a scaffold, implant or orthopedic regenerative construct for vascularized, critical sized bone defects which can quickly and efficiently generate an arterial blood flow throughout. This would allow for fast *in situ* delivery of blood, nutrients, progenitors and growth factors either through fluid perfusion or arterial grafting to the implant. The supply of pulsatile arterial blood through the proposed microchannels would then diffuse through the smaller, more biomimetic porous matrix, allowing for the development of vascularized bone tissue. Thus, we sought to evaluate preliminary hydrodynamic performance through our scaffold designs, to get a better idea of scaffold performance with regard to this desired effect on blood flow and body fluid introduction. The experimental setup has been successfully used for cardiovascular flow diagnostics under stenotic and stent-implanted conditions that are believed to occur in physiological and clinical environments [5,6]. We performed preliminary hydrodynamic measurements of pulsatile pressure and flow rates in this setup and compared them to a control case (see **Figures 2A-7E**). We hypothesize that microvascular mimicking structures that maintain temporal, pressure-flow rate phase shifts as those characterized in native blood vessels would constitute an effectively designed structure.

The pressure and flow rate waveforms have similar temporal profiles such as characteristic systolic and diastolic phases and intrinsic phase shifts or time delays. These phase shifts are certain instances very subtle and sensitive to the scaffold location; compare perforated lines in **Figures 2B, 2C** and **Figures 2D, 2E**. The scaled-channel designed structures, having large and small channels exhibited this phase shifting phenomenon, in a manner highly comparable and correlated to the clean artery

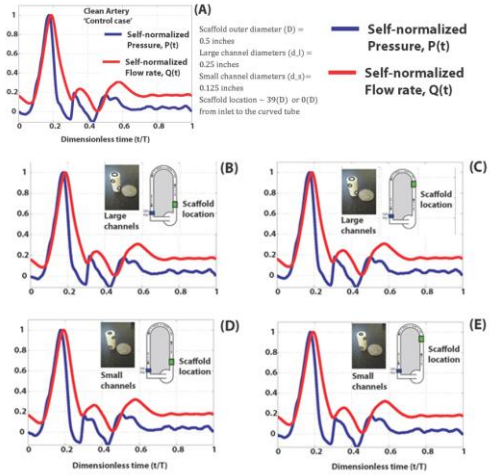

Figure 2 Experimental flow mechanics, pressure (blue) and flow rate (red) analysis of the (A) clean artery control, (B) Large channel models and (D) small channel models placed in a straight pipe and (C) large channel models and (E) small channel models placed in a curved pipe. All values are normalized for comparison, and are not the true measured experimental values.

–control case. This observation suggests that hydrodynamics of the designed micro-vessels may have the same characteristics as the control case representing native vessels with arterial blood flow. Accordingly, such designed structures may provide efficient and adequate blood and fluid transport in and out of the scaffold, without causing any flow disruptions to the system. Our future studies will rigorously evaluate these insights.

Scaffolds with smaller channels and nHA performed the best. Specifically this was due in part to adequate, yet more physiologically comparable fluid transfer, mechanical properties and both chemical and nanostructural contributions of the nHA. There was a slight decrease in collagen type I deposition on nHA scaffolds after 2 weeks of culture, but collagen type I content increased again after 3 weeks.

HUVEC adhesion showed good initial attachment on scaffold with small channels and conjugated nHA. However, 5 day proliferation, while showing good increase in cell number on all scaffolds, had a large spike on the large channel scaffolds without nHA after five days. When these scaffolds were imaged with a confocal microscope, they also showed the greatest density of cells, and the most developed vascular structures. This may have been for several reasons. All vascular endothelial cell types rely heavily on fluid flow and shear stresses to migrate, grow, and align to form new tissue. And while they also may leverage structural cues for growth, this is a dominant factor in development. Our scaffolds were cultured in static conditions, which may show different cell growth and vascular formation behavior, as compared to fluid flow conditions (such as in a bioreactor). However, it is important to consider a static condition, since our constructs would be initially implanted into a large bone defect, and not directly into blood vessels with available pumping arterial blood. The increased access to nutrients and chemicals in culture, and the potential for convective fluid mixing in scaffolds with larger microchannel spaces may have contributed to micro-shears in the fluid environment which could have effected cell growth. HUVECs are also a very potent cell type, and the presence of a softer substrate (no nHA) for cell attachment and more free space may have caused a high amount of HUVECs to grow more rapidly.

According to confocal imaging, all scaffolds showed highly enhanced hMSC attachment and spreading. 3D printed scaffolds displayed well integrated and highly aligned cell growth, displaying the effectiveness of these scaffolds to promote cellular organization. A decrease in size of our microchannels had denser, even more highly aligned cellular aggregates. Continued culture on scaffolds with small channels and conjugated nHA displayed increasing cell density, as well as larger and more spread cytoskeletons and filopodia. HUVECs which were also cultured on scaffold with small channels and nHA developed well after five days (**Figure 3**). Cells grew in dense, at times zig-zagging arrangements across scaffold features' surfaces. Despite the fact that larger, longer and more highly developed vascular structures could be seen on pure PLA scaffolds with large diameter channel networks, vascular growth was mostly only observed in the 3D printed channels, pores and between scaffolds features, whereas HUVECs could be seen growing on the full surface of nHA conjugated scaffolds. This demonstrated that nHA conjugated scaffold could direct efficient and effective growth and development within 3D printed structures.

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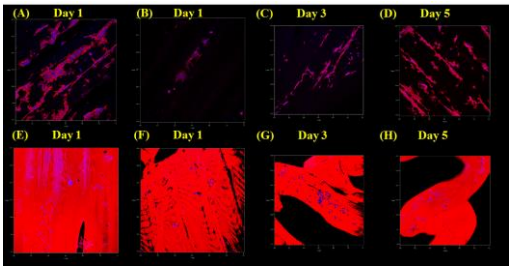

Figure 3 Confocal microcopy images of 1 day HUVEC growth on scaffolds with (A) 500 µm microchannels, (B) 250 µm microchannels, (E) 500 µm microchannels + nHA and VEGF, (F) 250 µm microchannels + nHA and VEGF. 3 and 5 day HUVEC growth on scaffolds with (C-D) 500 µm microchannels and (G-H) 250 µm microchannels + nHA + VEGF.

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