Published in final edited form as:

Biomacromolecules. 2010 March 8; 11(3): 682–689. doi:10.1021/bm901260y.

Enhanced Cell Ingrowth and Proliferation through Three-Dimensional Nanocomposite Scaffolds with Controlled Pore Structures

Kee-Won Lee[†], Shanfeng Wang[‡], Michael J. Yaszemski[†], and Lichun Lu^{†,*}

[†]Tissue Engineering and Biomaterials Laboratory, Departments of Orthopedic Surgery and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

[‡]Department of Materials Science and Engineering, The University of Tennessee, Knoxville, Tennessee 37996

Abstract

We present enhanced cell ingrowth and proliferation through crosslinked three-dimensional (3D) nanocomposite scaffolds fabricated using poly(propylene fumarate) (PPF) and hydroxyapatite (HA) nanoparticles. Scaffolds with controlled internal pore structures were produced from computer-aided design (CAD) models and solid freeform fabrication (SFF) technique, while those with random pore structures were fabricated by NaCl leaching technique for comparison. The morphology and mechanical properties of scaffolds were characterized using scanning electron microscopy (SEM) and mechanical testing, respectively. Pore interconnectivity of scaffolds was assessed using X-ray micro-computed tomography (micro-CT) and 3D imaging analysis. *In vitro* cell studies have been performed using MC3T3-E1 mouse preosteoblasts and cultured scaffolds in a rotating-wall-vessel bioreactor for 4 and 7 days to assess cell attachment, viability, ingrowth depth, and proliferation. The mechanical properties of crosslinked nanocomposite scaffolds were not significantly different after adding HA or varying pore structures. However, pore interconnectivity of PPF/HA nanocomposite scaffolds with controlled pore structures has been significantly increased, resulting in enhanced cell ingrowth depth 7 days after cell seeding. Cell attachment and proliferation are also higher in PPF/HA nanocomposite scaffolds. These results suggest that crosslinked PPF/HA nanocomposite scaffolds with controlled pore structures may lead to promising bone tissue engineering scaffolds with excellent cell proliferation and ingrowth.

Keywords

Poly(propylene fumarate) (PPF); Hydroxyapatite (HA); Nanocomposite; Solid freeform fabrication (SFF); Pre-osteoblast responses

Introduction

Tissue and organ regeneration by culturing and/or transplanting cell or tissue inside engineered scaffolds has become one of the most promising techniques for surgical therapy and biomedical research. The scaffolds support cell/tissue attachment, define the ultimate shape of the regenerated functional tissue, and guide tissue growth.¹ A number of synthetic polymers have been used as matrix materials for scaffolds because of the capability of controlling the properties such as surface chemistry, biodegradability, and mechanical strength. However,

*Corresponding author. Tel: (507) 538-4987. Fax: (507) 284-5075. lu.lichun@mayo.edu.

biocompatibility and cell-tissue interactions of these materials are less optimal as compared to natural biomaterials. To overcome these main limitations, combinations of synthetic and natural materials have been developed in various tissue engineering applications including skeletal tissue regeneration. Hydroxyapatite (HA), a major inorganic compound of human hard tissue, is considered an excellent candidate to enhance mechanical strength and osteoconductive properties.^{2–7} Although HA nanoparticles can strengthen the polymer matrix, the composite's mechanical properties depend mainly on the matrix. Furthermore, HA composition in the composite may not exceed a certain value to prevent disadvantages such as brittleness of the composites and poor torsional resistance. Therefore, it is crucial to choose a matrix material for achieving desired mechanical strength and modulus for load-bearing applications.

Poly(propylene fumarate) (PPF), an unsaturated linear polyester that can be crosslinked *in situ* to provide suitable mechanical properties, is one of the promising materials for load-bearing tissue regeneration.⁸ PPF has been used to form composites with enhanced mechanical strength and osteoconductive properties by adding calcium phosphates such as β -tricalcium phosphate (TCP).^{9,10} By incorporating HA nanoparticles, we have developed a series of crosslinkable nanocomposite disks and demonstrated that crosslinked PPF/HA nanocomposites have sufficient mechanical strength for bone tissue engineering, increased hydrophilicity and protein absorption on their surfaces with increasing HA contents, and enhanced 2D attachment and proliferation of pre-osteoblast *in vitro*.¹¹

Besides material properties, independent control of pore microstructure is a prerequisite for increasing cell growth into the three-dimensional (3D) scaffold and enhancing new tissue integration with the host. Several conventional techniques, such as gas foaming/particulate leaching,¹² phase separation,¹³ precipitation,¹⁴ and electrospinning,¹⁵ have been successfully used to fabricate polymer/HA nanocomposite scaffolds. However, these methods have limited controllability over the bulk properties of scaffolds such as porosity and average pore size, by varying parameters such as porogen size and content, temperature, and applied voltage. As an alternative method, solid freeform fabrication (SFF), including 3D printing,¹⁶ selective laser sintering,¹⁷ and indirect sacrifice mold technique,¹⁸ has improved the capability of controlling the microstructures of scaffolds by using computer-aided design (CAD). These studies have shown that controlling of individual pore size, shape, arrangement and interconnectivity within the scaffold is essential for 3D cell ingrowth and proliferation.

In this work, we have fabricated crosslinked PPF/HA nanocomposite scaffolds with two pre-designed pore structures from CAD models using SFF technique. For comparison, crosslinked PPF/HA nanocomposite scaffolds with random pore structures were fabricated using the conventional NaCl leaching technique. The structure, morphology, and mechanical properties of crosslinked PPF and PPF/HA nanocomposite scaffolds have been characterized using scanning electron microscopy (SEM) and mechanical testing. Pore interconnectivity of each scaffold was measured using micro-computed tomography (micro-CT) and image analysis. To investigate *in vitro* cellular responses, MC3T3-E1 mouse pre-osteoblasts were seeded on the scaffolds and cultured in a rotating-wall-vessel bioreactor for 4 and 7 days. Cell morphology, viability, ingrowth depth, and density were examined.

Experimental Section

PPF synthesis

All reagents were purchased from Aldrich Chemicals (Milwaukee, WI) and used as received, unless indicated otherwise. PPF was synthesized as described previously.¹⁹ Briefly, diethyl fumarate (DEF) and excess amount of 1,2-propylene glycol were polymerized together with hydroquinone (crosslinking inhibitor) and zinc chloride (catalyst) first at 100 °C for 1 hr and

then 150 °C for 7 hr to get the intermediate. Then the intermediate was transesterified to form PPF under vacuum at 150 °C for another 7 hr. Gel permeation chromatography (GPC) was used to determine the molecular weight and polydispersity of PPF. The GPC was carried out with a Waters 717 Plus Autosampler GPC system (Waters, Milford, MA) connected to a model 515 HPLC pump and model 2410 refractive index detector. Monodisperse polystyrene standards (Polysciences, Warrington, PA) with number average molecular weights (M_n) of 474, 6690, 18600, and 38000 g/mol were used to construct the calibration curve. The M_n and weight average molecular weights (M_w) of the synthesized PPF were 2104 and 3337 g/mol, respectively.

Scaffold modeling in CAD

Scaffold modeling was performed using 3D CAD software, SolidWorks (SolidWorks Corp., Concord, MA). Unit cell-based model was designed using two parameters (pore opening size and strut length) as a cubic block with cylindrical and spherical pore structures. To compare internal pore structures, pore opening size and strut length for each scaffold model were determined by calculating the ratio of pore opening size to strut length, corresponding to 55% of target porosity. Final models were created combining 27 unit cell models from the Boolean operation, and each pore of scaffold models was fully interconnected to the adjacent pores. External dimensions of all scaffold models were fixed as $5 \times 5 \times 5$ mm.

Scaffold fabrication using PPF/HA nanocomposites

HA nanoparticles were purchased from Berkeley Advanced Biomaterials (Berkeley, CA). The size range of HA nanoparticles is from 20 to 550 nm (average size = 100 nm) and their whiskers have long and short axis of ~100 and ~20 nm, respectively. PPF and HA nanoparticles were crosslinked by a free radical polymerization using benzoyl peroxide (BPO), 1-vinyl-2-pyrrolidinone (NVP), and *N*-dimethyl-*p*-toluidine (DMT) as free radical initiator, crosslinker, and accelerator, respectively. To prepare PPF/HA mixtures, 1 g of PPF and 0.4 g of NVP, corresponding to 40% NVP per gram of PPF, were mixed at 37 °C for 2 hr. HA nanoparticles were added to prepare PPF/HA nanocomposite with a HA composition of 30 wt.%. Forty microliters of initiator solution (50 mg of BPO in 250 μ L of NVP) and 16 μ L of accelerator solution (20 μ L of DMT in 980 μ L of methylene chloride) were added and mixed thoroughly. PPF/HA nanocomposite scaffolds with cylindrical and spherical pore structures were fabricated using 3D printing and injection molding technique.²⁰ Briefly, all CAD models were converted to stereolithography (STL) files, and then 2D sliced data (PTM) files with a 0.076mm of thickness using the ModelWorks software (SolidScape Corp., Merrimack, NH). The 3D phase-change ink jet printer, PatternMaster, was used to create 3D scaffolds for printing PTM files layer by layer with a build material (polystyrene, PS) and a support material (wax). PS was dissolved by immersing printed scaffolds into acetone for 30 min to obtain negative wax molds. Subsequently, this mold was put into a Teflon holder, and PPF/HA mixture was injected under 100 mmHg vacuum. To facilitate crosslinking, scaffolds were put into a convection oven at 40 °C for 1 hr. After crosslinking was completed, the scaffolds were separated from the Teflon holder and wax was dissolved in a cleaner solution (BIOACT VS-O, Petroferm Inc., Fernandina Beach, FL) at 40~60 °C for 1 hr. Finally, the scaffolds were dried completely at ambient temperature. For comparison, PPF scaffolds with pre-designed internal pore structures were fabricated from the same method without adding HA nanoparticles. On the other hand, PPF and PPF/HA nanocomposite scaffolds with random pore structures were fabricated using NaCl leaching. NaCl particles with sizes of 300–500 μ m and the calculated weight to reach a porosity of 55% were added to PPF and PPF/HA mixture. After initiator and accelerator solutions were added, the mixture was poured into rectangular Teflon molds with 12 holes ($5 \times 5 \times 8$ mm). The mold was placed in a convection oven at 60 °C for 1 h to facilitate crosslinking. Crosslinked PPF and PPF/HA scaffolds were removed from the mold and cut into cubes ($5 \times$

5 × 5 mm). Final scaffolds were put into water for 3–4 days to leach out NaCl particles. All the scaffolds were fabricated at the same time using the same PPF.

Scaffold morphology

Surface and cross-sectional morphology of PPF/HA nanocomposite scaffolds was examined by S-4700 cold-field emission scanning electron microscope (SEM) (Hitachi Instruments Inc., Japan). For cross-sectional images, scaffolds were frozen in liquid nitrogen and cut through the middle parallel to the horizontal plane. Scaffolds were mounted onto an aluminum stub, then sputter coated with gold-palladium. All samples were viewed at 3 kV accelerating voltage.

X-ray micro-computed tomography (micro-CT)

3D images of the scaffold were obtained from a custom-built micro-CT system.²¹ Scaffolds were mounted on a rotary stage and scanned in their entirety (approximately 160 mm³) at 17 keV of peak emission X-rays, being rotated 360° in 361 equiangular steps. Each of these 2D projections was deconvolved with its measured, spatially variant point spread function. All micro-CT images were reconstructed using a modified Feldkamp cone beam tomographic reconstruction algorithm²² to cubic voxels with 18 μm on each side. Images were segmented by an operator-selected threshold of intensities to separate voxels representing regions of differing density, and then analyzed to obtain volume fractions of each scaffold. Volume fractions of pores and polymer were obtained by selecting sub-regions of each volume representing a scaffold, and logging counts of voxels representing each material along the slices. Pore interconnectivity of each scaffold was assessed by performing morphological erosion followed by a dilation of the air space in all three dimensions, essentially blocking small pore connections.²³ One unit of dilation was 27 voxels and it closed off pores with a diameter of less than 36 μm because the voxel size was 18 μm³. Connected and isolated pore regions were then labeled, and finally the accessible void volume was calculated as the number of air voxels maintaining connections with the outside air as a percentage of the total air voxels. These quantitative analyses were implemented using the Analyze™ biomedical image analysis software (Biomedical Imaging Resources, Mayo Clinic, Rochester, MN).

Mechanical testing

Compression testing of PPF/HA nanocomposite scaffolds was conducted on an MTS 858 Mini Bionix II servohydraulic biaxial test system (MTS Systems Corp., Eden Prairie, MN). All samples were compressed at a rate of 1 mm/min up to a maximum force of 116 N. Ultimate stress and compressive modulus were determined as the maximum stress before failure and the slope of elastic region in the stress-strain curve, respectively.

Cell culture

MC3T3-E1 mouse pre-osteoblasts were cultured *in vitro* using Dulbecco's modified Eagle's medium (DMEM) F-12 (Sigma-Aldrich, St. Louis, MO), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA). PPF/HA nanocomposite scaffolds were sterilized in 70% ethanol for 24 h, washed in phosphate buffered saline (PBS; pH=7.4) several times, and pre-wetted in culture media for 24 h before cell seeding. Cells were seeded at a density of 200,000 cells per scaffold and cultured for 1 day in a humidified atmosphere of 5% CO₂ at 37 °C. After 1 day of static culture, all scaffolds were transferred into disposable vessels of a rotating wall vessel bioreactor (Synthecon Inc., Houston, TX) filled with 50 ml of media. Cells were cultured for 4 and 7 days in the bioreactor at 30 rpm rotating speed, to provide optimal balance of the centrifugal and gravitational forces.

Cell attachment and morphology

Cell morphology on PPF/HA nanocomposite scaffolds was examined at 24 hr post-seeding using SEM. Each scaffold was fixed with the Trump solution composed of 4% formaldehyde and 1% glutaraldehyde in PBS and washed using PBS and distilled water subsequently. For SEM imaging, the scaffolds were dehydrated with a graded series of ethanol (60, 70, 80, 95, and 100%), critical point dried, mounted onto an aluminum stub, and then sputter coated with gold-palladium. All samples were viewed at 3 kV accelerating voltage.

Cell viability and ingrowth depth

Cell viability and spatial distribution was examined by Live/Dead Viability/Cytotoxicity assay (Molecular Probes, Eugene, OR) at three time points (1, 4, and 7 days) by following the manufacturer's instructions. All scaffolds were scanned using the Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY) equipped with an argon/krypton ion laser for excitation at 488 and 568 nm. Optical sectioning was accomplished at 4 μ m thickness along the z-axis of the scaffold and all images captured were reconstructed into 3D images for analysis. Cell ingrowth depth of each scaffold was calculated by multiplying the total number of slices from top to bottom with the slice thickness of 4 μ m.

Cell proliferation

Cell density in PPF and PPF/HA nanocomposite scaffolds was determined using a fluorometric PicoGreen double-stranded DNA (dsDNA) quantification assay (Molecular Probes, Eugene, OR) at three time points by following the manufacturer's instruction. Total dsDNA content was measured by reading the absorbance at 408/530 nm (excitation/emission) with a microplate reader.

Statistical analysis

All measured data were reported as means $\pm$ standard deviations for $n = 4$. Student's *t*-test was performed using StatView software (SAS Institute, Cary, NC) to assess to assess statistically significant differences ($p < 0.05$).

Results and Discussion

PPF/HA nanocomposite scaffold fabrication

Table 1 shows the porosities and mean pore opening sizes of the fabricated scaffolds with three different internal pore structures measured using micro-CT and imaging analysis. Mean pore opening sizes and porosities of the actual fabricated scaffolds with two pre-designed pores showed variations from the CAD designs, but no significant differences were detected. To attain the same target porosity of 55%, cylindrical pore models with lower pore volume have larger pore opening size than spherical pore models. The ratio between pore opening size and strut length (L/D) in cylindrical pore models is consequently higher than that in spherical pore models given the same external dimensions of scaffold. Moreover, as the minimum printable feature size of the 3D printer is ~ 300 μ m, the pore opening size or strut length in CAD model design cannot be lower than this value and therefore pore opening size of spherical pore models was set as 301 μ m. All fabricated scaffolds have slightly lower porosity than the target value of 55%, which may occur in all fabrication steps or incomplete leaching of NaCl particles. Considering that complete porogen leaching is limited when thicker scaffolds are used with the conventional NaCl leaching technique, the exact target porosity of scaffolds with a thickness of 5 mm thick was difficult to obtain. These results demonstrate that all scaffolds with pre-designed microstructures were successfully fabricated by 3D printing and injection molding technique.

Scanning electron microscopy (SEM)

Morphology of crosslinked PPF and PPF/HA nanocomposite scaffolds with three internal pore structures were examined by SEM (Figure 1). In SEM images, scaffolds with two pre-designed internal pore structures have smooth surfaces and large-sized pores are fully interconnected in cross-sectional view, while scaffolds fabricated by NaCl leaching technique have irregular, rough surface and each pore has random cubic shape with different sizes. Scaffolds with spherical pores have larger pore volume than those with cylindrical pores despite their smaller pore opening sizes. It is noted that both pre-designed pores on the sides are larger than those on the top and bottom surfaces possibly because more overlapping pores are created in the sides during the layer-by-layer printing process. In contrast, scaffolds fabricated using NaCl leaching technique have random cubic pores with a variety of sizes as NaCl particles may be fused or isolated in the polymer matrix before leaching. This spatially non-uniform distribution of NaCl particles leads to a larger surface area ($494 \pm 33 \text{ mm}^2$) of scaffold than the two pre-designed pore structures (cylindrical: $234 \pm 19 \text{ mm}^2$, spherical: $341 \pm 18 \text{ mm}^2$), but decreased connections between adjacent pores that can be seen in cross-sectional view.

X-ray micro-computed tomography (micro-CT)

In 3D reconstructed images from micro-CT scans (Figure 2A), scaffolds with controlled pore structures showed that pores and struts in both top-bottom and side surfaces were consistently fabricated from CAD models. All pore structures are fully interconnected in all directions with a clear comparison with non-uniformly distributed and random cubic pore structure in the scaffolds fabricated using NaCl leaching technique.

Pore interconnectivity was quantified from iterative morphological dilation/erosion step and accessible void volume fraction (Figure 2B). Since total void voxels did not vary in each iteration, only connected void voxels are the main factor in determining the accessible void volume of each scaffold. Scaffolds with two pre-designed internal pore structures have larger accessible void volume than those with random pore structures and the difference increases with the number of iterations. To attain the same target porosity of 55%, cylindrical pore structures with a larger pore opening size have a slightly larger accessible void volume than spherical ones. Accessible void volume of scaffolds with spherical pores decreases abruptly when the minimum connection size is over $300 \mu\text{m}$, suggesting that accessible void volume is strongly correlated with the pore opening size. These results coincide with the different mean pore opening sizes between two pore structures shown in Table 1. Both accessible pore size and pore interconnectivity of synthetic porous biomaterials were found to relate to bone ingrowth and a minimum pore of over $100 \mu\text{m}^{25}$ or $300 \mu\text{m}^{26}$ was recommended to enhance bone formation and vascularization. Since most of these studies were based on implants and scaffolds with random pore structures, recent assessment of bone ingrowth using non-destructive 3D imaging technique shows that the size, length, and degree of interconnection are more crucial to tissue ingrowth rather than mean pore size.^{27–29}

In the scaffolds with random NaCl leached pores, accessible void volume decreased consistently with each iteration. At the minimum connection size of $288 \mu\text{m}$, scaffolds only have 20% of accessible void volume even though their mean pore opening size is $341 \pm 43 \mu\text{m}$. Comparing with the scaffolds with a mean spherical pore size of $317 \pm 22 \mu\text{m}$, most pores of the scaffolds fabricated using NaCl leaching technique lost the connections with adjacent pores, which resulted in isolated pores after dilation/erosion. These isolated pores still contribute to the apparent porosity of the scaffolds whereas no significant difference in porosity was found among three scaffolds; however, they do not contribute to the pore interconnectivity of the scaffolds as they decrease accessible void volume from outside air to the inside of the scaffolds. Besides increased isolate pores, pore connection paths of scaffolds fabricated using NaCl leaching technique are long, narrow, and tortuous because of spatially non-uniform distribution

of NaCl particles even when pores are connected with each other, which might hinder the access of nutrients or cells to the inside of the scaffolds through pores.

Mechanical testing

Mechanical strength of PPF/HA nanocomposite scaffolds increased slightly compared to that of PPF scaffolds, with no significant differences regarding HA composition and internal pore structures, which results may be attributed to the intrinsic rigid characteristics of crosslinked PPF itself. PPF used in this study has a high molecular weight and one crosslinkable segment in each repeat unit to ensure a rigid polymer network with a high density of crosslinking and consequently a high mechanical strength regardless of the inclusion of HA nanoparticles. In our previous study,¹¹ we have shown the average compressive modulus of 2D PPF disks was over 100 MPa. The ultimate stress and compressive moduli ranged from 216 ± 38 to 275 ± 18 MPa and from 31.4 ± 11 to 40.5 ± 5 MPa (Figure 3), respectively. Compared to the previously reported maximum compressive modulus (8.3 MPa) of poly(L-lactic acid) (PLLA)/HA nanocomposite scaffolds with the HA content of 50 wt.%,¹³ PPF/HA nanocomposite scaffolds in this study had a roughly 3.8 times higher compressive modulus with a lower HA content because of the high rigidity of crosslinked PPF. In addition, these values were close to the compressive modulus of 50~500 MPa of human trabecular bone.³⁰ It should be noted that the mechanical properties of PPF/HA nanocomposite scaffolds were more dependent on the intrinsic rigidity of PPF than the inclusion of HA and different pore internal structures.

Cell attachment and morphology

MC3T3-E1 mouse pre-osteoblasts were seeded onto PPF and PPF/HA nanocomposite scaffolds with three internal pore structures for 1, 4, and 7 days. SEM images of PPF and PPF/HA nanocomposite scaffolds with three internal pore structures 24 h after cell seeding are in Figure 4. Most cells attached to the edge of pores in two pre-designed pore structures or the corner of pores in the random NaCl leached pore structures, and spread out on the surface of the scaffolds. More connections between adjacent cells can be observed in PPF/HA nanocomposite scaffolds compared with PPF scaffolds, which is in agreement with previous studies for MC3T3 cell attachment on other polymer/HA nanocomposite scaffolds.^{12,17,31} From the previous study on 2D PPF/HA nanocomposite disks, we evidently demonstrated that the enhanced initial cell attachment could be attributed to increased hydrophilicity and protein absorption on the disk surface when increasing HA content.¹¹ However, no significant difference in initial cell attachment could be found regarding three internal pore structures of 3D scaffolds. Therefore, these results provided the evidence that the incorporation of HA nanoparticles to the crosslinked PPF enhanced the initial attachment of pre-osteoblast on the surface of scaffolds, which mainly affected the surface properties.

Cell viability and ingrowth depth

Cell viability and spatial distribution on PPF and PPF/HA nanocomposite scaffolds with three internal pore structures were examined using Live/Dead Viability/Cytotoxicity assay after 1 and 7 days. Most cells were viable in all scaffolds as seen in uniformly green color around the edge of pores (Figure 5). After 7 days of culture, cell density was higher in PPF/HA nanocomposite scaffolds compared with PPF scaffolds, indicated by the more intense green color in confocal laser scanning electron microscopy images. Unlike scaffolds with two pre-designed internal pore structures, more extensive distribution of live cells was seen in the scaffolds with random NaCl leached pores, which may result from larger surface area. Besides increased cell density, most live cells were distributed spatially along the pore wall. Especially, spatial distribution of live cells in the scaffolds was similar to their pre-designed internal pore structures such as cylindrical and spherical shape over a 7-day period of cell culture. This result provides the evidence that cells attached to the edge of pores first and then grew into the

scaffolds through the pores as guidance channels from surface to inside, and their spatial distribution in the 3D scaffold was determined by internal pore structures as a backbone.

Figure 5 (side view) and Figure 6 show cell ingrowth depth of PPF and PPF/HA nanocomposite scaffolds with three internal pore structures after 1, 4 and 7 days of culture using optical sectioning at a thickness of 4 μm . There were no significant differences regarding HA composition and internal pore structures at 1 day post-seeding. However, cell ingrowth depth significantly increased in the scaffolds with two pre-designed pores compared with those with NaCl leached random pores after 4 days post seeding. The effect of HA nanoparticles on cell ingrowth was only remarkable at 7 days post-seeding. Increased cell ingrowth was more affected by the pore internal structure than the incorporation of HA and strongly related to increased pore interconnectivity represented as accessible void volume fraction in Figure 2B. The maximum cell ingrowth depths in the PPF/HA nanocomposite scaffolds were $336 \pm 2.8 \mu\text{m}$ for cylindrical pores, $300 \pm 3.6 \mu\text{m}$ for spherical pores, and only $168 \pm 3.8 \mu\text{m}$ for random NaCl leached pores, respectively. Compared with the maximum penetration depth of osteoblasts (190~220 μm) in previous scaffolds with 150~710 μm of NaCl leached pores after 56 days,³² our results suggest that pre-designed and interconnected pore structures support more cell growth into the scaffold at a relatively short culture period under the dynamic culture condition. For the two pre-designed pore structures, the scaffolds with cylindrical pores had slightly larger cell ingrowth depth than those with spherical pores. The straightly aligned cylindrical pores without variation in pore sizes from inside to outside may lead to a higher mean velocity and permeability of water flow through the pores than the spherical pores. This result is consistent with an earlier finding that cylindrical pore design is more permeable than spherical one at same pore volume fraction.³³ Besides internal pore shapes, the pore opening size is another factor for the difference in cell ingrowth depth because the scaffolds with cylindrical pores had larger mean pore opening size than those with spherical pores given the same porosity, as shown in Table 1. Nevertheless, the scaffolds with two pre-designed pore structures have similar fully interconnected pores with the minimum opening size of over 300 μm compared to those with random NaCl leached pores. In agreement with previous studies, the central aligned cylindrical pores with their interconnection size of 300~500 μm significantly increased the infiltration of osteoblast cells and penetration depth of mineralized tissue into the scaffolds.^{34–36}

Cell proliferation

Cell proliferation on PPF and PPF/HA nanocomposite scaffolds was quantified by the total dsDNA content over a 7-day period (Figure 7). There were no significant differences in the total dsDNA content regarding HA content and internal pore structure at 1 day post-seeding. However, the total dsDNA content was much higher on PPF/HA nanocomposite scaffolds than that on PPF scaffolds after 4 days post seeding regardless of pore structures. The effects of two pre-designed pore structures on cell density were obvious at 7 days post-seeding, revealing significantly increased total dsDNA contents compared to random NaCl leached pores. These results demonstrated that the incorporation of HA nanoparticles could enhance the proliferation of pre-osteoblast in PPF/HA nanocomposite scaffolds as well. The present study will help to understand *in vivo* bone formation in the 3D scaffolds with controllable structural features and the delivery of bone morphogenetic protein when incorporated with the PPF/HA scaffolds.

Conclusions

Crosslinked PPF/HA nanocomposite scaffolds with two pre-designed internal pore structures have been successfully fabricated by 3D printing and injection molding. These scaffolds have fully interconnected pore networks, leading to significantly larger accessible void volume fraction, than those with random NaCl leached pores. No significant differences in ultimate

stress and compressive modulus were found regarding HA composition and internal pore structures due to the intrinsic high rigidity of PPF matrix after crosslinking. *In vitro* cell studies using MC3T3 cells cultured in a rotating-wall-vessel bioreactor up to 7 days showed that PPF/HA nanocomposite scaffolds enhanced initial cell attachment at 24 hr and proliferation at 4 and 7 days post-seeding. In addition, two pre-designed pore structures enhanced cell growth inside the scaffolds, demonstrating increased cell ingrowth depth after 4 days of culture compared to those with random NaCl leached pores. This study not only reveals the role of HA nanoparticles in enhancing cell proliferation in the crosslinked PPF/HA nanocomposite scaffolds, but also indicates the importance of pore interconnectivity in the scaffolds with pre-designed pore structures in influencing cell ingrowth.

Acknowledgments

This study was funded by the Mayo Foundation and National Institutes of Health (R01 AR45871 and R01 EB003060). The authors would like to acknowledge James A. Gruetzmacher for PPF synthesis and James E. Tarara for confocal laser scanning microscope imaging. The authors also thank Dr. Erik L. Ritman's laboratory for 3D image analysis, especially Patricia E. Beighley and Jorn Op Den Buijs for micro-CT scanning and image processing.

References

1. Hutmacher DW. *Biomaterials* 2000;21:2529. [PubMed: 11071603]
2. Hollinger JO, Battistone GC. *Clin. Orthop. Relat. Res* 1986;207:290. [PubMed: 3522015]
3. Burg KJL, Porter S, Kellam JF. *Biomaterials* 2000;27:2347. [PubMed: 11055282]
4. Singh S, Ray SS. *J. Nanosci. Nanotechnol* 2007;7:2596. [PubMed: 17685274]
5. Dimitrievska S, Petit A, Ajji A, Bureau MN, Yahia L. *J. Biomed. Mater. Res. A* 2008;84:44. [PubMed: 17600325]
6. Xiao X, Liu R, Huang Q. *J. Mater. Sci. Mater. Med* 2008;19:3429. [PubMed: 18574674]
7. Wang S, Kempen DH, Yaszemski MJ, Lu L. *Biomaterials* 2009;30:3359. [PubMed: 19339048]
8. Wang S, Lu L, Yaszemski MJ. *Biomacromolecules* 2006;7:1976. [PubMed: 16768422]
9. Peter SJ, Kim P, Yasko AW, Yaszemski MJ, Mikos AG. *J. Biomed. Mater. Res* 1999;44:314. [PubMed: 10397934]
10. Peter SJ, Lu L, Kim DJ, Mikos AG. *Biomaterials* 2000;21:1207. [PubMed: 10811302]
11. Lee KW, Wang S, Yaszemski MJ, Lu L. *Biomaterials* 2008;29:2839. [PubMed: 18403013]
12. Kim SS, Ahn KM, Park MS, Lee JH, Choi CY, Kim BS. *J. Biomed. Mater. Res. A* 2007;80:206. [PubMed: 17072849]
13. Wei G, Ma PX. *Biomaterials* 2004;25:4749. [PubMed: 15120521]
14. Wei J, Li Y. *Eur. Polym. J* 2004;40:509.
15. Ito Y, Hasuda H, Kamitakahara M, Ohtsuki C, Tanihara M, Kang IK, Kwon OH. *J. Biosci. Bioeng* 2005;100:43. [PubMed: 16233849]
16. Simon JL, Rekow ED, Thompson VP, Beam H, Ricci JL, Parsons JR. *J. Biomed. Mater. Res. A* 2008;85:371. [PubMed: 17688275]
17. Wiria FE, Leong KF, Chua CK, Liu Y. *Acta Biomater* 2007;3:1. [PubMed: 17055789]
18. Chu TM, Hollister SJ, Halloran JW, Feinberg SE, Orton DG. *Ann. N. Y. Acad. Sci* 2002;961:114. [PubMed: 12081877]
19. Wang S, Lu L, Gruetzmacher JA, Currier BL, Yaszemski MJ. *Macromolecules* 2005;38:7358.
20. Lee KW, Wang S, Lu L, Jabbari E, Currier BL, Yaszemski MJ. *Tissue Eng* 2006;12:2801. [PubMed: 17518649]
21. Jorgensen SM, Demirkaya O, Ritman EL. *Am. J. Physiol* 1998;275:H1103. [PubMed: 9724319]
22. Feldkamp LA, Goldstein SA, Parfitt AM, Jasion G, Kleerekoper M. *J. Bone Miner. Res* 1989;4:3. [PubMed: 2718776]
23. Moore MJ, Jabbari E, Ritman EL, Lu L, Currier BL, Windebank AJ, Yaszemski MJ. *J. Biomed. Mater. Res. A* 2004;71:258. [PubMed: 15376269]

24. Sikavitsas VI, Bancroft GN, Mikos AG. J. Biomed. Mater. Res 2002;62:136. [PubMed: 12124795]
25. Hulbert SF, Young FA, Mathews RS, Klawitter JJ, Talbert CD, Stelling FH. J. Biomed. Mater. Res 1970;4:433. [PubMed: 5469185]
26. Karageorgiou V, Kaplan D. Biomaterials 2005;26:5474. [PubMed: 15860204]
27. Otsuki B, Takemoto M, Fujibayashi S, Neo M, Kokubo T, Nakamura T. Biomaterials 2006;27:5892. [PubMed: 16945409]
28. Jones AC, Arns CH, Sheppard AP, Hutmacher DW, Milthorpe BK, Knackstedt MA. Biomaterials 2007;28:2491. [PubMed: 17335896]
29. van Lenthe GH, Hagenmuller H, Bohner M, Hollister SJ, Meinel L, Muller R. Biomaterials 2007;28:2479. [PubMed: 17258316]
30. Murugan R, Ramakrishna S. Compos. Sci. Technol 2005;65:2385.
31. Chou YF, Dunn JC, Wu BM. J. Biomed. Mater. Res. B Appl. Biomater 2005;75:81. [PubMed: 16001421]
32. Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, Mikos AG. Biomaterials 1998;19:1405. [PubMed: 9758040]
33. Hollister SJ. Nat. Mater 2005;4:518. [PubMed: 16003400]
34. Chang BS, Lee CK, Hong KS, Youn HJ, Ryu HS, Chung SS, Park KW. Biomaterials 2000;21:1291. [PubMed: 10811311]
35. Frosch KH, Barvencik F, Lohmann CH, Viereck V, Siggelkow H, Breme J, Dresing K, Sturmer KM. Cells Tissues Organs 2002;170:214. [PubMed: 11919409]
36. Rose FR, Cyster LA, Grant DM, Scotchford CA, Howdle SM, Shakesheff KM. Biomaterials 2004;25:5507. [PubMed: 15142732]

Figure 1. Scanning electron micrographs of scaffolds with three different internal pore structures. Top: 3D view, middle: cross-sectional view, and bottom: magnification of the dotted box of middle images. Scale bars represent 600 μm (top and middle) and 500 μm (bottom).

Figure 2. Micro-CT scan and 3D image analysis of scaffolds with three different internal pore structures. (A) CAD models and 3D reconstruction images of scaffolds obtained from the micro-CT scan. Top: solid part and bottom: pore part. Scale bars represent 1 mm. (B) Accessible void volume as an indication of pore interconnectivity calculated from iterative dilation/erosion operations.

Figure 3. Mechanical properties of PPF and PPF/HA nanocomposite scaffolds with three different internal pore structures.

Figure 4. Scanning electron micrographs of MC3T3 pre-osteoblasts at 24 h post-seeding onto PPF and PPF/HA nanocomposite scaffolds with three different internal pore structures. Scale bars represent 30 μm.

Figure 5.

Confocal laser scanning microscopy images of MC3T3 pre-osteoblasts cultured on PPF and PPF/HA nanocomposite scaffolds with three different internal pore structures for 1, 4, and 7 days and stained by Live/Dead viability/cytotoxicity assay. White asterisks represent pores. Scale bars represent 300 μm .

Figure 6.

Cell ingrowth depth of PPF and PPF/HA nanocomposite scaffolds with cylindrical, spherical, and random NaCl leached pores at three different time points. * $p < 0.05$ compared to values of scaffolds with random NaCl leached pores. † $p < 0.05$ compared to values of PPF scaffolds.

Figure 7. Total dsDNA of PPF and PPF/HA nanocomposite scaffolds with cylindrical, spherical, and random NaCl leached pores at three different time points. *, $\diamond$ $p < 0.05$ compared to values of PPF scaffolds. † $p < 0.05$ compared to values of PPF/HA scaffolds with random NaCl leached pores.

Table 1
Porosities and mean pore opening sizes of fabricated scaffolds with three different internal pore structures.

Pore shape	L/D ^[a]	Strut length (D) (μm)	Pore opening size (μm)		Porosity (%)	
			Design (L)	Scaffold ^[c]	Design	Scaffold ^[c]
Cylinder	3.07	300	920	903 (± 26)	55	52.4 (± 2.3)
Sphere	0.45	670	301	397 (± 22)	55	53.1 (± 2.9)
Random cube	-	-	300~500 ^[b]	341 (± 43)	55	52.9 (± 4.8)

^[a] Pore opening size/strut length in CAD design.

^[b] The size of NaCl particles.

^[c] Standard deviations are given in parentheses (*n* = 4).