INVITED SUBMISSION

Three-Dimensional Printing of Tissue Engineering Scaffolds with Horizontal Pore and Composition Gradients

Luis Diaz-Gomez, PhD,¹⁻³ Panayiotis D. Kontoyiannis,¹⁻³
Anthony J. Melchiorri, PhD,¹⁻³ and Antonios G. Mikos, PhD¹⁻³

This work investigated a new three-dimensional (3D) printing methodology to prepare porous scaffolds containing horizontal pore and composition gradients. To achieve that, a multimaterial printing technology developed in our laboratory was adapted to incorporate pore gradients. Fibers were printed by welding segments with unique material compositions and fiber diameters. Particularly, we focused on the preparation of model composite poly(ϵ -caprolactone)-based scaffolds with radial gradients of particulate hydroxyapatite (HA) content (higher concentrations in the outer region of the scaffold) and porosity (higher in the inner region). The morphology of the scaffolds revealed that the methodology allowed the fabrication of discrete regions with compressive mechanical properties similar to human trabecular bone while maintaining structural integrity. HA distribution was homogeneous within individual regions and no particle aggregation was detected by microCT analysis and Alizarin Red S staining. Finally, the incubation of the scaffolds in simulated body fluid resulted in the deposition of significantly higher amounts of calcium deposits in the regions of the scaffolds with higher HA content. This work provides a new tool for the preparation of porous scaffolds containing porosity and composition gradients for complex tissue engineering applications.

Keywords: 3D printing, gradient scaffold, tissue engineering, biofabrication, multimaterial printing

Impact Statement

In this study, we report the development of a novel multimaterial segmented three-dimensional printing methodology to fabricate porous scaffolds containing discrete horizontal gradients of composition and porosity. This methodology is particularly beneficial to preparing porous scaffolds with intricate structures and graded compositions for the regeneration of complex tissues. The technique presented is compatible with many commercially available bioprinters commonly used in biofabrication, and can be adapted to better replicate the architectural and compositional requirements of individual tissues compared with traditional scaffold printing methods.

Introduction

TISSUE ENGINEERING OFFERS a wide variety of new strategies for bone regeneration.^{1,2} Among these developing strategies, the fabrication of three-dimensional (3D) porous scaffolds using biodegradable materials is a popular method of recapitulating specific properties found in bone tissue.³ The structure of human bone consists of cortical (80% by mass) and trabecular (20% by mass) bone, with proportions that differ depending on the location.⁴ Cortical bone presents a low porosity (near 10%) with voids filled with osteocytes, canaliculi, and blood vessels. In contrast, trabec-

ular bone presents a higher porosity (50–90%), resulting in compressive properties an order of magnitude lower than cortical bone.⁵

An ideal 3D scaffold for bone engineering should exhibit similar mechanical and structural properties to the targeted tissue and location.^{6,7} The extent and quality of new bone formation within these scaffolds depend strongly on their pore size, porosity, and interconnectivity, both as a function of structural permeability and mechanics, as well as material composition.⁸ Specifically, scaffolds intended for bone regeneration should present a highly porous interconnected structure to allow nutrient diffusion, cell migration, and

¹Department of Bioengineering, BioScience Research Collaborative, Rice University, Houston, Texas.

²Biomaterials Laboratory, Rice University, Houston, Texas.

³NIH/NIBIB Center for Engineering Complex Tissues.

angiogenesis, while incorporating osteoconductive moieties that support tissue ingrowth and mineralization.⁹

Recently, the introduction of 3D printing in tissue engineering has allowed the development of intricate structures with high definition and reproducibility due to precise control over the deposition of the printed materials.¹⁰ Our laboratory has reported the preparation of porous scaffolds containing vertical and horizontal gradients of composition for bone and osteochondral regeneration.^{11,12} Printing pore gradients by altering pore arrangement and size can mimic native tissue function, particularly in bone. Previous studies revealed the effect of pore size and channel geometry in the osteogenic differentiation of mesenchymal stem cells using 3D printed porous scaffolds containing gradients of varying pore sizes.^{13,14}

However, to enhance bone formation, it is also important that scaffolds match the mechanical and compositional features of bone tissue. This requires the incorporation of material gradients. For example, introducing hydroxyapatite (HA) gradients to enhance mineralization in dense regions like cortical bone while maintaining lower ceramic content in the inner regions to simulate the more diffuse composition of trabecular bone could improve tissue formation.¹⁵

The aim of this work was the development of a 3D printing technology based on multimaterial segmental printing to prepare dual gradient scaffolds, containing discrete material compositions and pore sizes by printing segments of different compositions and widths. The validation and reproducibility of the technique was assessed in terms of the ability to mimic different architectures and compositions within the same scaffold similar to those of controls prepared with uniform compositions and pore sizes. It was also hypothesized that this printing methodology is suitable for the fabrication of scaffolds with architectures resembling the bone tissue by reproducing the graded porosity and transition between cortical and trabecular bone. We assessed if the graded structures could mimic the different mechanical behavior of trabecular and cortical bone within distinct regions of the scaffold. Finally, we evaluated the effect of the graded composition on the mineralization of the scaffold.

Materials and Methods

Materials

Poly(ϵ -caprolactone) (PCL; nominal molecular weight 50 KDa) was obtained from Polysciences (Warrington, PA). HA (particle size: 886 nm), sodium hydrogen carbonate

(NaHCO₃), potassium chloride (KCl), di-potassium hydrogen phosphate trihydrate (K₂HPO₄·3H₂O), magnesium chloride hexahydrate (MgCl₂·6H₂O), tris(hydroxymethyl) aminomethane ((CH₂OH)₃CNH₂), and Alizarin Red S were from Sigma-Aldrich (St. Louis, MO). Calcium chloride (CaCl₂) was from Fisher Scientific (Waltham, MA). Sodium chloride (NaCl) was from TJ Baker (Center Valley, PA). Sodium sulfate (Na₂SO₄) was from VWR (Radnor, PA). All the chemicals were used as received.

Scaffold printing

Printing materials were prepared from PCL and HA mixtures in different concentrations, as shown in Table 1. Powdered components were homogeneously mixed using a mortar and pestle, placed in printer cartridges, heated to 160°C, and extruded using a 3D Bioplotter (EnvisionTEC, Gladbeck, Germany). In this study, three printing heads were used, each one loaded with a different composition.

Three-dimensional models for printing were designed in SketchUp (Trimble, Sunnyvale, CA). The printing model for gradient scaffolds containing three regions consisted of a cylinder 4.7 mm in diameter surrounded by two concentric rings (7.4 and 10 mm in diameter, respectively) overlapping by 0.4 mm as shown in Figure 1. Scaffolds containing two regions were printed using a 3D model consisting of a cylinder 5.5 mm in diameter surrounded by a concentric ring of 10 mm in diameter. A 3D model containing two regions was used to evaluate the reproducibility and resolution of the printing methodology. Controls were printed from a single cylinder model of 10 mm in diameter.

All the scaffolds used in this study were printed with 12 layers and a height of 4 mm. The printing pattern consisted of noncontinuous parallel straight fibers with a 0.6 mm on-center spacing and each new layer was deposited perpendicular to the previous layer. As a result, each fiber was composed of welded segments of different compositions, following a methodology previously developed in our laboratory.¹¹ Different pore sizes were achieved by varying the diameter of the segments using 22G (small pores), 24G (medium pores), or 27G (large pores) extruding needles. The control of the height of the fibers printed with different needle diameters (22G, 24G, or 27G) was achieved by adjusting the needle offset to 0.26 mm.

The layer height corresponded to the thickness of the smallest fiber, which resulted in larger fibers printed with a

TABLE 1. COMPOSITION AND PRINTING CONDITIONS OF COMPOSITE SCAFFOLDS

Scaffold	PCL (wt%)	HA (wt%)	Needle gauge	Pressure (bar)	Speed (mm/s)	Preflow (s)	Postflow (s)
PCL	100	0	22	4.5	1.3	0.3	0.3
			24	5.0	1.2	0.3	0.3
			27	5.5	1.0	0.7	0.5
PCL-HA15	85	15	22	4.5	1.2	0.3	0.3
			24	5.0	0.5	0.3	0.3
			27	5.5	0.8	0.7	0.5
PCL-HA30	70	30	22	5.0	1.0	0.3	0.3
			24	5.5	0.8	0.7	0.7
			27	6.5	0.5	1.0	0.5

HA, hydroxyapatite; PCL, poly(ϵ -caprolactone).

FIG. 1. 3D model dimensions (*left*) and filling pattern (*right*) of the dual graded scaffolds containing three regions with unique compositions and pore sizes. *Orange*, *blue*, and *red* colors represent different compositions and fiber diameters. 3D, three-dimensional.

flattened shape and allowed the printing of scaffolds without any limitation in the total height of the construct. Homogeneous control scaffolds (PCL, PCL-HA15, and PCL-HA30) and graded scaffolds, containing three regions (outer ring: PCL-HA30, middle ring: PCL-HA15, inner region: PCL); and two regions (outer ring: PCL-HA30, inner region: PCL) were fabricated under specific extrusion printing conditions (pressure, speed, pre- and postflow) as shown in Table 1.

Physical characterization of 3D scaffolds

The fiber and pore size, porosity, and interconnectivity of the composite scaffolds were analyzed using microCT and confirmed by scanning electron microscopy (SEM). Briefly, samples from all experimental groups were scanned using a SkyScan 1272 X-ray MicroCT (Bruker, Kontich, Belgium). A volume of interest (VOI) fitting the area of each region was drawn. Interconnectivity is reported as the percentage of voids accessible from outside the VOI (threshold 7 μm). For comparative purposes, fiber and pore sizes were evaluated by SEM. Scaffolds were sputter coated with gold and imaged using SEM (Quanta 400 ESEM FEG; FEI, Hillsboro, OR). Samples were imaged under high vacuum at 10 kV and different magnifications.

Mechanical characterization

The tensile strength of individual fibers was evaluated to determine the strength and structural integrity of the welding between the different segments of the noncontinuous printed fibers. Briefly, single fibers (15 mm length) printed from welded PCL-HA30, PCL-HA15, and PCL segments with an average width of 760, 493, and 291 μm underwent tensile testing using an Enduratec ELF 3200 tensile bench (Bose, Framingham, MA) and were evaluated at 1 mm/s until 100% strain.

The compressive mechanical properties of the scaffolds ($n=5$) were measured using a mechanical testing bench (858 MiniBionixII[®]; MTS, Eden Prairie, MN; 10 kN load cell). Scaffolds were compressed perpendicularly to the printing plane at a crosshead speed of 1 mm/min after an initial preload of 25 N until 20% strain was reached.

Analysis of mineralization

The incubation of scaffolds in simulated body fluid (SBF) was conducted to analyze the chemical and morphological changes in the samples by mimicking interactions in the physiological environment. SBF has ion concentrations

comparable to those present in human blood plasma. The SBF solution was prepared following the method described by Kokubo and Takadama.¹⁶ Briefly, NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, hydrochloric acid (HCl), CaCl₂, Na₂SO₄, and (CH₂OH)₃CNH₂ were dissolved sequentially in Milli-Q water. After sterile filtering, the pH was adjusted to 7.4 using 1M HCl. The freshly prepared solution was kept at 4°C until use. Scaffolds were placed individually in 15 mL conical tubes and incubated in SBF for 3 weeks at 37°C. The SBF solution was changed every other day. After the samples were dried in a vacuum chamber at room temperature, they were characterized using SEM and energy-dispersive X-ray spectroscopy (EDX).

The presence of HA on the surface of the scaffolds was visualized by means of Alizarin Red staining. Briefly, scaffolds were incubated in SBF for 7 days and medium was changed every other day. Then, the SBF solution was discarded and the scaffolds were gently washed with water. Each scaffold was incubated in 5 mL of 40 mM Alizarin Red S solution for 5 min, washed thoroughly with water, and then imaged using a brightfield microscope (Leica, Germany).

Statistical analyses

Statistical analyses were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA). All results were expressed as mean values with standard deviations. One-way analysis of variance and Tukey's multiple comparison posttest were used. Differences were considered significant for $p < 0.05$.

Results

Validation of the printing method

In this work, we developed a biofabrication method involving the deposition of consecutive segments of discrete compositions and diameters to form continuous fibers and, subsequently, scaffolds with precise and distinct regions with different compositions and porosities. The method used in this work was adapted from a multimaterial printing methodology developed in our group recently.¹¹ Figure 2 shows the different steps in the preparation of the dual gradient scaffolds. Briefly, a scaffold containing an inner region of PCL-HA30 was printed by depositing fibers of 250 μm in diameter. In a second step, a middle ring was created by printing segments of PCL (450 μm in diameter) following those previously deposited to form the inner

FIG. 2. Printing methodology developed for the preparation of dual graded scaffolds. First, the *inner region* segments (PCL-HA30) were printed using a 27G needle. Then, the *middle ring* segments (PCL) were printed with a 24G needle. Finally, the *outer ring* segments (PCL-HA15) were printed using a 22G needle. Each layer was printed with fibers rotated 90° compared with the previous layer. HA, hydroxyapatite; PCL, poly(ϵ -caprolactone).

region using an overlap of 0.4 mm to ensure proper welding of the different segment compositions. For the outer ring, fibers of PCL-HA15 (750 μ m in diameter) were deposited following the same procedure as for the middle ring. This procedure results in the creation of continuous fibers containing up to three segments with discrete compositions and diameters (and, consequently, pore sizes).

After printing consecutive layers alternating the fiber orientation 90°, the resulting scaffold contains distinct regions of compositions and porosities. The tensile behavior of the welded fibers was evaluated using a tensile bench. The force versus displacement was monitored until 100% strain (Supplementary Fig. S1). The results showed the continuous stretching of the welded fibers without any critical failure and confirming the high integrity of the constructs printed using the segmental printing methodology.

The reproducibility of the printing methodology was evaluated by printing a dual gradient of compositions (a PCL inner region and PCL-HA30 outer ring) and porosities for the preparation of porous scaffolds of 10 and 4 mm in diameter. The results (Table 2 and Fig. 3) demonstrated the feasibility of the printing methodology to produce scaffolds with two distinct radial regions with porosities and compositions relevant to bone regeneration. Specifically, scaffolds with 4 and 10 mm in diameter showed homogeneous HA distributions in the ceramic-containing regions. Similarly, fiber width and pore size presented standard deviations

lower than 10% in all cases. MicroCT reconstructions revealed the homogeneous distribution of HA within the different regions of the scaffolds and no aggregation was observed in those compositions, including HA at 15 or 30 wt%. SEM micrographs confirmed proper welding between the segments of different compositions and fiber widths (Fig. 4). Despite the composition and fiber width used, the welding was only noticeable by the small artifacts resulting from the needle offset after the printing of each segment. However, these artifacts had no significant effect on the structure of the gradient scaffolds compared with the controls prepared using continuous printing.

Preparation and characterization of dual gradient scaffolds

After the reproducibility and resolution of the methodology were validated, scaffolds containing three radial regions were printed to create interconnected porous structures with a radial gradient of porosity and composition. A gradient scaffold was designed mimicking a bone structure containing a PCL inner region with a porosity similar to trabecular bone (60–70%), a PCL-HA15 transition ring (50% porosity), and a PCL-HA30 outer ring with a porosity similar to cortical bone (10–20%). Control scaffolds prepared with uniform compositions (PCL, PCL-HA15, and PCL-HA30) and porosities (20%, 50%, and 65%) were also

TABLE 2. STRUCTURAL CHARACTERIZATION OF DUAL-GRADED SCAFFOLDS CONTAINING TWO DISTINCT REGIONS WITH SPECIFIC POROSITIES AND COMPOSITIONS WAS ASSESSED BY TAKING 30 RANDOM MEASUREMENTS OF EACH SCAFFOLD PER GROUP ($N=3$ SCAFFOLDS IN EACH GROUP)

<i>Scaffold (diameter)</i>	<i>Composition</i>	<i>Porosity (%)</i>	<i>Fiber size (μm)</i>	<i>Pore size (μm)</i>
Gradient 1 (10 mm)	Outer ring (PCL-HA15)	17 $\pm$ 4	826 $\pm$ 21	201 $\pm$ 19
	Middle ring (PCL)	48 $\pm$ 7	449 $\pm$ 30	522 $\pm$ 22
	Inner region (PCL-HA30)	66 $\pm$ 5	265 $\pm$ 13	690 $\pm$ 23
Gradient 2 (10 mm)	Outer ring (PCL-HA30)	27 $\pm$ 5	616 $\pm$ 16	343 $\pm$ 14
	Inner region (PCL)	63 $\pm$ 6	313 $\pm$ 27	660 $\pm$ 16
Gradient 3 (4 mm)	Outer ring (PCL-HA30)	17 $\pm$ 5	841 $\pm$ 33	246 $\pm$ 21
	Inner region (PCL)	62 $\pm$ 10	267 $\pm$ 26	546 $\pm$ 48
Gradient 4 (4 mm)	Outer ring (PCL-HA30)	42 $\pm$ 9	456 $\pm$ 32	470 $\pm$ 18
	Inner region (PCL)	75 $\pm$ 7	244 $\pm$ 20	533 $\pm$ 32

FIG. 3. Transaxial images generated from microCT reconstructions of multimaterial scaffolds prepared with two or three specific regions. (A, E) represent a gradient scaffold 10 mm in diameter containing three regions (PCL-HA30 in the inner region, PCL in the middle ring, PCL-HA15 in the outer ring; Gradient 1). (B, F) represent a gradient scaffold 10 mm in diameter containing two regions (PCL in the inner region and PCL-HA30 in the outer ring; Gradient 2). (C, D, G, and H) represent scaffolds of 4 mm in diameter prepared with PCL in the inner region and PCL-HA30 in the outer ring (Gradient 3 and Gradient 4). HA isolated from the microCT reconstructions (E–H) revealed the homogeneous distribution of ceramic microparticles within each region of the scaffolds without the presence of noticeable aggregations. Scale bar = 500 μm (A, B, E, and F); 250 μm (C, D, G, and H).

printed using a simple continuous extrusion printing method. Gradient scaffolds were printed using the segmented printing methodology described in the validation of the printing method section. The aim was to precisely recreate the structural features measured for the control scaffolds in each unique region of the gradient scaffold while maintaining high interconnectivity and good structural integrity.

The structure of the printed gradient scaffolds was evaluated by microCT (Figs. 5 and 6). Each segment of the gradient scaffolds presented uniform and homogeneous pore sizes and fiber widths and were continuously aligned with the major axes of each adjacent segment. The interconnectivity obtained for pores larger than 7 μm was over 97% for all the scaffolds evaluated. The method proved to be very reproducible of the morphology when pore size, fiber width, and porosity of scaffolds were compared with the respective control scaffold. Specifically, the outer region of the gradient

scaffolds (PCL-HA30, small pore size) presented a pore size and fiber width of 284 ± 30 and 748 ± 28 μm , respectively, which was statistically similar to the small pore control scaffolds. Similarly, the pore size and fiber width measured for the middle ring (PCL-HA15, medium pore size) were 529.7 ± 16 and 440 ± 25 μm , respectively. These values were also not significantly different from the PCL-HA15 control (pore size: 526 ± 26 μm ; fiber width: 453 ± 20 μm). Finally, the inner region containing PCL (large pore size) presented a pore size of 671 ± 26 μm and a fiber width of 272 ± 32 μm , also statistically similar to the corresponding PCL control (pore size: 679 ± 21 μm ; fiber width: 291 ± 23 μm).

Each region of the gradient scaffolds presented a similar porosity compared with the controls and in all cases the interconnectivity was close to 100% (threshold was 7 μm). The obtained porosities for the distinct regions of the gradient scaffolds were calculated from the microCT reconstruction of

FIG. 4. SEM micrographs showing the morphology of the surface of the different regions of the 10 mm in diameter gradient scaffolds at the welding points between segments of different compositions and fiber widths. (A) Highlights the welding area in gradient scaffold prepared with two distinct regions (PCL in the inner region and PCL-HA30 in the outer ring); (B, C) The welding areas of a gradient scaffold containing three regions. (B) Welding area between the PCL-HA30 inner region and the PCL middle ring. (C) Welding area between the PCL middle ring and the PCL-HA15 outer ring. Scale bar = 500 μm .

FIG. 5. Cross-sectional images of (A) PCL, (B) PCL-HA15, (C) PCL-HA30, and (D) gradient (*outer ring*: PCL-HA30, *middle ring*: PCL-HA15, and *inner region*: PCL) composite scaffolds generated from a 3D reconstruction through microCT. Scale bar = 500 μm .

the scanned scaffolds. A VOI was defined with the same dimensions of the 3D model for each. The outer ring presented a porosity of 16.8%, similar to the PCL-HA30 control (17.5%). The middle ring (PCL-HA15; 47.5%) and the inner region (PCL; 66.3%) also presented porosities similar to their respective controls (47.5% and 66.1%, respectively).

The mechanical properties of the gradient scaffolds and controls were tested to evaluate the effect of composition and pore size (Fig. 7). Mechanical compression was carried out on a compressive bench using scaffolds with uniform porosity and composition as controls. The results showed that the scaffolds with the smallest porosity (scaffolds with a

pore size similar to the outer ring of the gradient scaffolds) had the highest compressive modulus, in the range of 180–230 MPa. The incorporation of HA in the composition led to a significant increase in the compressive modulus compared with the PCL scaffolds. The scaffolds with the medium pore size showed compressive moduli in the 105–125 MPa range. PCL-HA30 scaffolds showed a significant increase in the compressive mechanical properties, compared with the PCL scaffolds. Finally, scaffolds with the highest porosity (large pores) led to compressive modulus values significantly lower, in the 30–40 MPa range. The incorporation of HA to the composition of the scaffolds with large pores led to a

FIG. 6. Fiber width, pore size, and porosity of individual regions of 3D gradient scaffolds containing specific compositions and pore sizes, and controls prepared with single compositions and pore sizes (small, medium, or large pores). Results showed no statistical differences in the structure between the controls and regions of the gradient scaffolds with the structural features. Each composition and pore size are represented as mean $\pm$ standard deviation ($n=5$). Regions of the gradient scaffolds were individually analyzed: PCL (gradient *inner*), PCL-HA15 (gradient *middle*), and PCL-HA30 (gradient *outer*) compositions. Different letters denote a statistical difference ($p < 0.05$).

FIG. 7. Compressive modulus of controls and gradient (outer ring: large fiber width of PCL-HA30; middle ring: medium fiber width of PCL-HA15; and inner region: small fiber width of PCL) 3D scaffolds. Data reported as mean $\pm$ standard deviation ($n=5$ scaffolds). Different letters denote a statistical difference ($p < 0.05$).

significant increase in the compressive modulus of the composite scaffolds proportional to the amount of HA in the composition. The gradient scaffolds designed with an outer ring of PCL-HA30, a middle ring of PCL-HA15, and an inner region of PCL led to compressive modulus *ca.* 130 MPa, similar to the medium pore size scaffolds.

The effect of scaffold composition on the mineralization was evaluated using SEM/EDX after 3 weeks of incubation in SBF (Fig. 8 and Supplementary Fig. S2). Gradient scaffolds and controls (PCL, PCL-HA15, and PCL-HA30) were immersed in 10 mL of SBF and incubated at 37°C. Ac-

cording to the SEM micrographs, PCL scaffolds and the PCL regions of the gradient scaffolds showed no presence of deposited calcium after 21 days in SBF. All compositions tested showed strong background C and O peaks in the EDX analysis, which can be attributed to the presence of PCL as the main component of the scaffolds. PCL-HA15 regions in the gradient scaffold and their corresponding controls showed few aggregates of CaP deposits on the surface of the scaffolds after 21 days in SBF, compared with day 0. On the other hand, a thin apatite layer was observed for PCL-HA30 regions and controls after 21 days in SBF. The presence of sharp peaks of Ca and P in the PCL-HA30 region of the dual gradient scaffolds revealed that the calcium phosphate layer observed in the SEM images corresponds to the mineral deposition from the SBF media. The Ca:P ratios for the PCL-HA15 and PCL-HA30 regions of the gradient scaffolds after 21 days were 1.51 and 1.45, respectively.

The mineral deposition on the surface of the gradient scaffolds after incubation in SBF was also assessed by Alizarin red S staining (Supplementary Fig. S3). The calcium present on the surface of the scaffolds was stained in red when exposed to the Alizarin red S staining for 20 min. Gradient scaffolds that were not incubated in SBF were used as controls to determine the calcium present on the surface of the scaffolds due to their ceramic content. According to the obtained images, PCL-HA15 regions showed a lower density of red spots compared with PCL-HA30, where the presence of stained calcium on the surface of the scaffolds was more evident. PCL scaffolds showed no presence of any stained calcium, as expected. After 7 days in SBF, PCL and PCL-HA15 regions showed a similar concentration of calcium deposits as in day 0. However, PCL-HA30 regions

FIG. 8. SEM images of the control surface [(A) PCL, (B) PCL-HA15, and (C) PCL-HA30] and gradient [(D) inner region/PCL; (E) middle ring/PCL-HA15; and (F) outer ring/PCL-HA30] scaffolds revealed the presence of calcium deposits (indicated by white arrows) on the surface of the scaffolds containing HA 15 wt% (middle ring gradient scaffold) and HA 30 wt% (control and outer ring gradient scaffold) after incubation in SBF for 21 days. Scale bar = 20 μ m. EDX representative spectra of gradient scaffolds (G: inner region/PCL; H: middle ring/PCL-HA15; and I: outer ring/PCL-HA30) after 21 days of incubation in SBF. EDX, energy-dispersive X-ray spectroscopy; SBF, simulated body fluid.

(outer ring of the scaffolds) showed an extensive presence of calcium on the surface of the scaffolds due to the high deposition of minerals during the incubation in the SBF medium compared with day 0.

Discussion

In this work, a new printing technique was developed for the fabrication of scaffolds with radial gradients in composition and pore size by modification of our previously described multimaterial segmented printing methodology.¹¹ Radial gradients were achieved by printing fibers composed of welded segments with discrete compositions and widths.

This printing method allowed for the preparation of layer-independent structures with high interconnectivity and controlled porosity and composition without introducing weak points in the welding between segments that could lead to a decrease in the integrity of the scaffolds. Compared with previously described printing methodologies for the preparation of porous scaffolds containing pore gradients,¹³ the technology presented in this work enables the fabrication of porous scaffolds with an interconnectivity close to 100%. The high interconnectivity relies not only on the pores within the same region but in the entire structure, as evidenced from the analysis of the structure. This high interconnectivity is necessary for cell migration and fluid exchange through the different regions of the scaffolds and is also essential for neovascularization from surrounding tissues after implantation *in vivo*.^{17–19} Furthermore, the versatility of this printing method allows scaffolds to be prepared with precise patterns without altering the arrangement or alignment of the fibers at the interface between different regions since it is not necessary to deposit fibers transversally with respect to the previous region. Fibers can be fabricated in this manner to simulate the extrusion of a continuous and well-integrated structure.

The methodology presented in this study can only be reproduced when the height of each layer is adjusted by the height of the thinnest fiber. During printing, there are no gaps in the height of each region due to the difference in the thickness of the fibers. This adjustment on the height of each layer results in the flattening of the thicker fibers caused by the needle offset without affecting the pore size or interconnectivity of the scaffold. Furthermore, the resulting uniform height of the layers allows for the possibility to build without any height restriction. Most of the commercially available printers work with printing resolutions in the 100–150 μm range. Such resolutions allow the printing of fibers using this methodology with sufficient height clearance to create vertical pores with sizes adequate to allow horizontal fluid exchange and cell migration.²⁰

An additional advantage of the printing methodology described in this study is the possibility to incorporate gradients of composition. This can be accomplished in continuous fibers while combining a pore gradient with material gradients. These gradients can be accurately reproduced without sacrificing the structural integrity of the fibers. Despite the interest of the multimaterial printing reported by our laboratory showing potential to locally control the composition and degradation of the scaffolds, the introduction of dual gradients of pore and composition enhances the applications of the printing methodology.

In this study, we used bone as our target tissue because of the lack of alternatives to fabricate scaffolds with precise architectures, compositions, and mechanical properties similar to the natural tissue without compromising the structural and mechanical integrity of the implant.²¹ PCL was used as the main component due to its well-documented printability, biocompatibility, biodegradability, and consistent mechanical properties.^{22,23} HA was included due to its osteoconductivity, biodegradability, and the reinforcement of the mechanical properties when incorporated in composite materials.²⁴ Furthermore, nano- and micrometric particles of HA dispersed in polymeric matrices can be extruded without inhibiting printing due to clogging or aggregation effects using commercial 3D printers.^{25,26} Accordingly, the 3D reconstructions of the control scaffolds and gradients obtained using microCT showed HA micro-particles homogeneously distributed within the scaffolds (controls) or regions (gradients) and no aggregation was observed in any condition.

The printing parameters and conditions used in this study are in the working range of a wide variety of commercially available printers with interchangeable heads. Furthermore, the printing speed is not a limiting factor since it is possible to print a scaffold of 10 mm in diameter and 3 mm high containing three distinct regions with specific pore size and composition in less than 20 min using a printer with three heads and no intricate setups. The printing speed and automation of the process may make dual gradient technology commercially attractive and easy to scale up.

As shown in the methodology validation, the printing technique was useful in the preparation of scaffolds with two or three different compositions and pore sizes. This demonstrates the adaptability of the technique for different biological structures and locations. Furthermore, it was possible to create 4 mm scaffolds with two distinct regions and an inner region as small as 1.2 mm in diameter, which is crucial to overcome size limitations when using small animal models.²⁷ The printing methodology described in this study can be adapted to the requirements of the tissue to be engineered. Simply by changing the printing tips used and/or the space between printed fibers, it is possible to accommodate virtually any overall porosity values and pore sizes to mimic the different architectures of native bones. The characterization of the obtained scaffolds showed the highly precise ability of the printing method to reproduce the compositional and structural features of distinct control scaffolds.

One of the main objectives of the study was to reproduce the specific porous structures found in different regions of the bone tissue, namely cortical bone, with a porosity lower than 20% and trabecular bone, with porosities above 50%, depending on the location.⁵ Additionally, we introduced HA gradients as a strategy to potentially promote the formation of cortical bone (in those regions with higher HA density) or trabecular bone (more diffused ceramic content since it is less mineralized than cortical bone due to more intense tissue remodeling²⁸). As shown in the structural analysis, the gradient scaffolds presented a graded porosity with values in the range of cortical bone for the outer ring (PCL-HA30, porosity *ca.* 17%), and in the range of trabecular bone for the middle ring (PCL-HA15, *ca.* 50% porosity) and inner region (PCL, *ca.* 67% porosity). Furthermore, the

architecture of each region showed no statistical differences compared with the controls printed with homogeneous porosity and composition, demonstrating the suitability of the printing methodology to reproduce specific architectures within the same scaffold. This allows the technique to be adapted to other tissues or applications when gradients of compositions and/or porosities are required, such as graded tissues or interfaces.^{21,29,30}

The improvement in the compressive strength when different amounts of HA were incorporated in the composition was evident in the control scaffolds with large pores. Higher ceramic content in the composition resulted in an increase of the compressive modulus of the composite scaffolds, proportional to the HA present in the composition.³¹ The presence of HA in the composition of the control scaffolds with medium and small pore sizes led to statistical differences in the compressive modulus compared with the PCL scaffolds, but these differences were not shown when scaffolds prepared with different proportions of HA (15 or 30 wt%) were compared. This was likely due to the already high modulus of the PCL in geometries with low porosities resulting in little or no effect detected in the mechanical bench testing.³²

While the incorporation of microscale particles of HA has resulted in an increase in the compressive modulus of scaffolds, we have previously reported that the inclusion of nanoscale HA particles does not result in this same effect.^{11,33} The reinforcement effect of micrometric HA particles when incorporated in polymeric scaffolds has been previously reported.²⁴ Gradient scaffolds showed a compressive modulus of 126.2 ± 7.6 MPa, similar to the values obtained for the medium fiber width PCL-HA30 control (115.3 ± 9.1 MPa) and in the range of human trabecular bone (50–150 MPa).¹⁸ As expected, the compressive modulus of the gradient scaffolds was an approximate average between the modulus of the individual regions. Simply by altering the material and composition, the compressive modulus of printed scaffolds presented in this study can be altered by up to an order of magnitude. This enables the fabrication of regions with significantly different mechanical behaviors to better mimic complex natural tissues.

As previously reported,^{34,35} the incorporation of HA in the PCL scaffolds resulted in an increase of the deposition of calcium on the surface of the scaffolds after incubation in SBF. As described previously, mineralization of PCL scaffolds usually requires the pretreatment of the surface of the materials using plasma treatment or other such techniques.³⁶ In gradient scaffolds, the apatite layer formed on the surface of the PCL-HA30 region was significantly larger compared with the dispersed aggregates observed for the PCL-HA15 region. Furthermore, the calcium-deficient apatite layer observed on the surface of the regions containing HA presented a Ca:P ratio smaller compared with the 1.67 stoichiometric ratio of HA.²³ In contrast, no CaP aggregates were observed on the region with only PCL. Apatite deposition on the surface of scaffolds after incubation in SBF has been previously related to an increase of the proliferation and differentiation of osteoblasts and also to the prediction of *in vivo* bone bioactivity.³⁷ These findings demonstrate the potential of this printing method to incorporate regions with different osteoconductive properties within the same scaffold.

Conclusions

In this work, we developed and evaluated a new printing methodology to prepare porous scaffolds containing interconnected regions with distinct compositions and porosities. The printing methodology supported the formation of fibers composed of welded segments with discrete compositions and widths. The mechanical integrity of the welded fibers was validated with tensile testing. We printed and characterized scaffolds consisting of regions with different PCL/HA ratios and porosities. The morphological analysis confirmed that the printing technique is suitable to prepare scaffolds containing predesigned regions with varied compositions, porosities with high resolution and fidelity, and high interconnectivity between pores. Compressive mechanical properties were found to be dependent on the HA content and pore size, and were in the range of human trabecular bone. Finally, the incubation of the scaffolds in SBF for 21 days revealed a significant mineralization in the region of the scaffolds containing higher proportions of HA in the composition. The work described in this study provides a robust printing methodology for the reproducible fabrication of scaffolds with intricate geometries and graded compositions for the regeneration of complex tissues.

Acknowledgments

This work was supported by the National Institutes of Health (P41 EB023833). L.D.-G. acknowledges Consellería de Cultura, Educación e Ordenación Universitaria for a Post-doctoral fellowship (Xunta de Galicia, ED481B 2017/063).

Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3

References

1. Liu, J., and Kerns, D.G. Mechanisms of guided bone regeneration: a review. *Open Dent J* **8**(Suppl 1), 56, 2014.
2. Garg, T., and Goyal, A.K. Biomaterial-based scaffolds—current status and future directions. *Expert Opin Drug Deliv* **11**, 767, 2014.
3. Agarwal, R., and García, A.J. Biomaterial strategies for engineering implants for enhanced osseointegration and bone repair. *Adv Drug Deliv Rev* **94**, 53, 2015.
4. Brandi, M.L. Microarchitecture, the key to bone quality. *Rheumatology* **48**(suppl 4), 3, 2009.
5. Sikavitsas, V.I., Temenoff, J.S., and Mikos, A.G. Biomaterials and bone mechanotransduction. *Biomaterials* **22**, 2581, 2001.
6. Polo-Corrales, L., Latorre-Esteves, M., and Ramirez-Vick, J.E. Scaffold design for bone regeneration. *J Nanosci Nanotechnol* **14**, 15, 2014.
7. O'Brien, F.J. Biomaterials & scaffolds for tissue engineering. *Mater Today* **14**, 88, 2011.
8. Hannink, G., and Arts, J.J.C. Bioresorbability, porosity and mechanical strength of bone substitutes: what is optimal for bone regeneration? *Injury* **42**, S22, 2011.

9. Zadpoor, A.A. Bone tissue regeneration: the role of scaffold geometry. *Biomater Sci* **3**, 231, 2015.
10. Derakhshanfar, S., Mbeleck, R., Xu, K., Zhang, X., Zhong, W., and Xing, M. 3D bioprinting for biomedical devices and tissue engineering: a review of recent trends and advances. *Bioact Mater* **3**, 144, 2018.
11. Diaz-Gomez, L., Smith, B.T., Kontoyiannis, P.D., Bittner, S.M., Melchiorri, A.J., and Mikos, A.G. Multimaterial segmented fiber printing for gradient tissue engineering. *Tissue Eng Part C Methods* **25**, 12, 2019.
12. Trachtenberg, J.E., Placone, J.K., Smith, B.T., Fisher, J.P., and Mikos, A.G. Extrusion-based 3D printing of poly(propylene fumarate) scaffolds with hydroxyapatite gradients. *J Biomater Sci Polym Ed* **28**, 532, 2017.
13. Di Luca, A., Longoni, A., Criscenti, G., Mota, C., van Blitterswijk, C., and Moroni, L. Toward mimicking the bone structure: design of novel hierarchical scaffolds with a tailored radial porosity gradient. *Biofabrication* **8**, 045007, 2016.
14. Kim, K., Dean, D., Wallace, J., Breithaupt, R., Mikos, A.G., and Fisher, J.P. The influence of stereolithographic scaffold architecture and composition on osteogenic signal expression with rat bone marrow stromal cells. *Biomaterials* **32**, 3750, 2011.
15. Nyberg, E.L., Farris, A.L., Hung, B.P., *et al.* 3D-printing technologies for craniofacial rehabilitation, reconstruction, and regeneration. *Ann Biomed Eng* **45**, 45, 2017.
16. Kokubo, T., and Takadama, H. How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* **27**, 2907, 2006.
17. Leong, K.F., Cheah, C.M., and Chua, C.K. Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials* **24**, 2363, 2003.
18. Turnbull, G., Clarke, J., Picard, F., *et al.* 3D bioactive composite scaffolds for bone tissue engineering. *Bioact Mater* **3**, 278, 2018.
19. Zhang, X., Chang, W., Lee, P., *et al.* Polymer-ceramic spiral structured scaffolds for bone tissue engineering: effect of hydroxyapatite composition on human fetal osteoblasts. *PLoS One* **9**, e85871, 2014.
20. Murphy, C.M., Haugh, M.G., and O'Brien, F.J. The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. *Biomaterials* **31**, 461, 2010.
21. Miao, X., and Sun, D. Graded/gradient porous biomaterials. *Materials (Basel)* **3**, 26, 2009.
22. Chia, H.N., and Wu, B.M. Recent advances in 3D printing of biomaterials. *J Biol Eng* **9**, 4, 2015.
23. Bose, S., Roy, M., and Bandyopadhyay, A. Recent advances in bone tissue engineering scaffolds. *Trends Biotechnol* **30**, 546, 2012.
24. Dorozhkin, S.V. Bioceramics of calcium orthophosphates. *Biomaterials* **31**, 1465, 2010.
25. Jariwala, S.H., Lewis, G.S., Bushman, Z.J., Adair, J.H., and Donahue, H.J. 3D printing of personalized artificial bone scaffolds. *3D Print Addit Manuf* **2**, 56, 2015.
26. Wang, K., Ho, C.-C., Zhang, C., and Wang, B. A review on the 3D printing of functional structures for medical phantoms and regenerated tissue and organ applications. *Engineering* **3**, 653, 2017.
27. McGovern, J.A., Griffin, M., and Huttmacher, D.W. Animal models for bone tissue engineering and modelling disease. *Dis Model Mech* **11**, dmm033084, 2018.
28. Oftadeh, R., Perez-Viloria, M., Villa-Camacho, J.C., Vaziri, A., and Nazarian, A. Biomechanics and mechanobiology of trabecular bone: a review. *J Biomech Eng* **137**, 010802, 2015.
29. Seidi, A., Ramalingam, M., Elloumi-Hannachi, I., Ostrovidov, S., and Khademhosseini, A. Gradient biomaterials for soft-to-hard interface tissue engineering. *Acta Biomater* **7**, 1441, 2011.
30. Loh, Q.L., and Choong, C. Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size. *Tissue Eng Part B Rev* **19**, 485, 2013.
31. Huang, B., Caetano, G., Vyas, C., Blaker, J., Diver, C., and Bártolo, P. Polymer-ceramic composite scaffolds: the effect of hydroxyapatite and β -tri-calcium phosphate. *Materials (Basel)* **11**, 129, 2018.
32. Hajiali, F., Tajbakhsh, S., and Shojaei, A. Fabrication and properties of polycaprolactone composites containing calcium phosphate-based ceramics and bioactive glasses in bone tissue engineering: a review. *Polym Rev* **58**, 164, 2018.
33. Bittner, S.M., Smith, B.T., Diaz-Gomez, L., *et al.* Fabrication and mechanical characterization of 3D printed vertical uniform and gradient scaffolds for bone and osteochondral tissue engineering. *Acta Biomater* **90**, 37, 2019.
34. Kepa, K., Coleman, R., and Grøndahl, L. In vitro mineralization of functional polymers. *Biosurf Biotribol* **1**, 214, 2015.
35. Urquia Edreira, E.R., Wolke, J.G.C., Jansen, J.A., and van den Beucken, J.J.J.P. Influence of ceramic disk material, surface hemispheres, and SBF volume on in vitro mineralization. *J Biomed Mater Res Part A* **103**, 2740, 2015.
36. Jeon, H., Lee, H., and Kim, G. A surface-modified poly(ϵ -caprolactone) scaffold comprising variable nanosized surface-roughness using a plasma treatment. *Tissue Eng Part C Methods* **20**, 951, 2014.
37. Qi, X., Pei, P., Zhu, M., *et al.* Three dimensional printing of calcium sulfate and mesoporous bioactive glass scaffolds for improving bone regeneration in vitro and in vivo. *Sci Rep* **7**, 42556, 2017.

Address correspondence to:

Antonio G. Mikos, PhD
Department of Bioengineering, MS-142
BioScience Research Collaborative
Rice University
6500 Main Street
Houston, TX 77030

E-mail: mikos@rice.edu

Received: April 19, 2019

Accepted: May 24, 2019

Online Publication Date: July 10, 2019