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ORIGINAL ARTICLE

Developing 3D Scaffolds in the Field of Tissue Engineering to Treat Complex Bone Defects

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Abstract

Polymers have been extensively used to develop 3D scaffolds in the field of tissue engineering and consist of certain design requirements such as biocompatibility, structural properties, and varying porosity inside of complex geometries, all with the ultimate goal of incorporating living cells within the scaffold structure. In this work, we present the synthesis and material characterization of hybrid spools using polycaprolactone (PCL) as the base polymer. We demonstrate that a commercial 3D Fused Deposition Modeling printer such as MakerBot can be used to print 3D scaffolds using three types of polymer spools: PCL, PCL-poly lactic acid, and PCL-hydroxyapatite. Data derived from computer tomography can be used to develop hollow porous cages using PCL. Finally, we demonstrate that log-pile scaffolds are capable of being infused with a mixture of living cells and gelatin hydrogel and that high cellular viability is maintained throughout the printed structure. This work could be potentially useful in the treatment of patients with complex bone defects.

Introduction

BONE HAS EMERGED as the second most transplanted tissue in the world as a result of patient traumas, aging populations, osteoporosis, and the prevalence of bone tumors.[1] Since current clinical therapies have not been sufficiently successful, the field of bone tissue engineering continues to develop new bone substitutes by combining porous biomaterial scaffolds with relevant cells and growth factors. Important aspects of bone tissue engineering include the fabrication of porous polymer scaffolds with patient-specific geometries, the necessary structural strength to house living cells, and the ability to facilitate tissue ingrowth during *in vitro* development of bone tissue or during *in vivo* implantation.[2–6]

To promote cell proliferation, tissue growth, and remodeling, porous scaffolds have been developed using several different manufacturing approaches. The use of traditional fabrication methods such as solvent casting, freeze-drying, porogen leaching, fiber bonding, dual-phase separation, and gas foaming, typically create simple geometries and only allow limited control over pore interconnectivity within 3D scaffolds, both of which are extremely essential for bone tissue applications.[7–9] With the goal of developing scaffolds with a 100% pore interconnectivity, additive manufacturing and other free form fabrication techniques such as laser sintering,[10] stereolithography,[11] and Fused Deposition Modeling (FDM)[3,12] have been used.[5,6] Several researchers have developed manufacturing equipment based on FDM principles where a polymer spool is extruded through a heated nozzle and can subsequently be moved using commands obtained from a computer-aided manufacturing program.[12,13]

While most laboratories have typically developed custom-made extrusion-based equipment to print user-defined 3D scaffolds, the emergence of easy-to-use commercial 3D printers has allowed other researchers with nonmanufacturing backgrounds to print complex 3D models. For example, physicians and clinicians use MakerBot printers to develop anatomically accurate physical models for educational purposes and to practice surgeries on physical models. The drawback, however, is that these physical models can be only built using commercially available and easy-to-use polymer spools. Biomaterial spools such as polycaprolactone (PCL), a thermoplastic commonly used for bone tissue engineering applications,[14] are not commercially available and have to be custom made in research laboratories. These direct-writing techniques that rely on the properties of colloidal biomaterial inks are able to be used to print droplets, hot-melts, or continuous filament printing techniques,[15–19] but need specialized manufacturing knowledge to operate them. Moreover, incorporation of living cells within 3D printed scaffolds is yet another significant challenge and cells seeded on scaffolds with small pore sizes typically result in uneven cellular

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distribution throughout the scaffold, with most cells adhering to the periphery of the scaffold.

In this work, we demonstrate the synthesis and fabrication of 3D scaffolds using hybrid PCL spools using an easy-to-use commercial MakerBot 3D printer. Hybrid spools were developed by mixing fillers such as poly-l-lactic-acid (PLA) or hydroxyapatite (HA) particles within the PCL matrix, following which mechanical and materials properties were characterized. We also demonstrate the incorporation of living cells into the interior of the log-pile scaffold with a facile and easy-to-use technique.

Materials and Methods

Development of hybrid spools

Spools were made using a Randcastle microfilament extruder (Randcastle, Inc.) and a base material of PCL pellets having a 3 mm diameter and 70–90 kDa molecular weight (Sigma Aldrich) (Fig. 1A). The raw PCL material and the fillers, either HA (Sigma Aldrich) or PLA, were mixed and fed into the extruder's hopper. The shear force of the machine's extrusion screw fed the raw material (PCL) and filler (either PLA or HA) into the four heating zones, allowing for homogenization of PCL-PLA composites and mixing of PCL-HA composites. A cooling zone was located below the third heating zone and the thread of the screw was reversed, thereby causing a buildup of pressure that forced the melted polymer mixture out of the head (heating zone 4). The temperatures were kept at 160°F (72°C), just above 140°F or 60°C, which was the melting point for PCL, for the pure PCL spool to keep the polymer in liquid state during the extrusion process and maintain constant pressure. For the PCL-HA spool, the temperatures were raised to 170°F (76.6°C) to allow HA powder to mix with liquid PCL, and for the PCL-PLA spool, the temperatures were raised to 320°F (160°C) to melt both of the polymers since PLA has a melting point between 300° and 320°F. The pressure for each spool was kept at 1000 psi ± 200 psi and fed through a custom nozzle with an opening diameter of 1.57 mm. The filament expanded once it left the extruder head to the appropriate 1.75 ± .05 mm so that it could be wound into spools and fed into the commercially available 3D printer, MakerBot Replicator 2 (MakerBot Industries) (Fig. 1B).

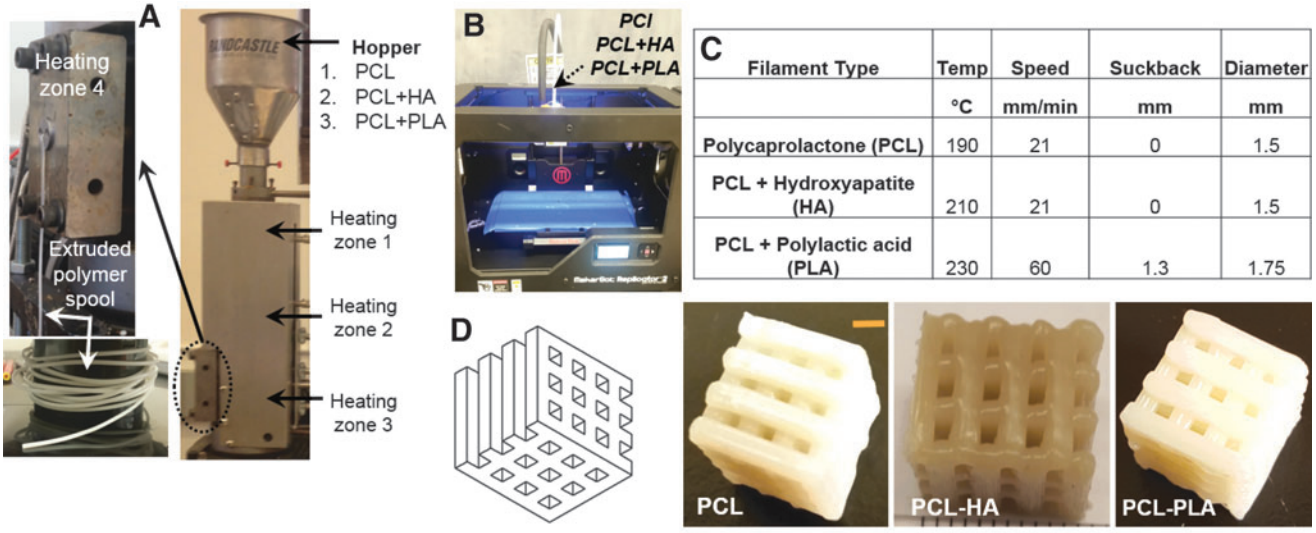
Chemical composition and testing

To determine the final material composition postextrusion and printing, a high-resolution thermogravimetric analysis (TGA) was performed on PCL, PCL-HA, and PCL-PLA samples using a Q500 thermogravimetric analysis (TA Instruments). The TGA heated spool samples to and beyond their degradation points while weighing them so that the amounts of each polymer/component could be determined based on weight percentages.

Three-dimensional printing and mechanical testing

Spools were loaded into the MakerBot and several different types of software were used to generate the .stl files used to make the g-code for the log-pile scaffolds. SolidWorks (Dassault Systemes) was used to make the log-pile geometry with two different sizes: (1) 10.5 mm × 10.5 mm × 12 mm and (2) 6 mm^3, with sufficient porosity to obtain repeatability and mechanical reliability. In addition to log-pile scaffolds, two complex geometries were fabricated using a computer tomography (CT) scan of a right femur. An .stl file was downloaded from thingiverse.com, and subsequently added to Meshmixer (Autodesk), a program that allows for unique customization of .stl's to use different mesh generating tools to make user-specified designs.

To test the mechanical strength of the scaffolds, the larger log-pile structures (Fig. 1) were placed between two plates on a compression MTS machine (Sintech 2G) with a 10K Newton load cell. The samples were compressed to failure



Filament Type	Temp	Speed	Suckback	Diameter
	°C	mm/min	mm	mm
Polycaprolactone (PCL)	190	21	0	1.5
PCL + Hydroxyapatite (HA)	210	21	0	1.5
PCL + Polylactic acid (PLA)	230	60	1.3	1.75

FIG. 1. **(A)** The raw material is fed into the filament extruder creating a long strand of three polymer spools (PCL, PCL-HA, and PCL-PLA). **(B)** A commercially available MakerBot Replicator 3D printer. **(C)** The printer settings are set for each filament spool, respectively. **(D)** The model of the log-pile scaffolds and the printed counterparts to the right. Scale bar: 2 mm. HA, hydroxyapatite; PCL, polycaprolactone; PLA, polylactic acid. Color images available online at www.liebertpub.com/3dp

and the machine recorded the downward cross head travel and the force from the load cell.

Cell incorporation within log-pile scaffolds

A mixture of gelatin methacrylate (GelMA) mixed with human osteosarcoma cells (Saos-2) was incorporated within log-pile scaffolds. Using a previously reported protocol,[20,21] GelMA was synthesized. Briefly, porcine skin gelatin was mixed at 10% (w/v) in phosphate-buffered saline (PBS; Thermo Fisher Scientific), stirred at 45°C, and methacrylic anhydride was added to the solution and stirred for 3 h. After stirring, the solution was dialyzed against distilled water for 1 week (40°C), freeze-dried, and stored at −80°C until needed. For cell encapsulation experiments, a final GelMA prepolymer solution of 7% was prepared by mixing freeze-dried GelMA with PBS and 0.25% UV photoinitiator Irgacure 2959 (Specialty Chemicals) at room temperature.

Human osteosarcoma cells (Saos-2) were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modification of Eagle's media (DMEM; Life Technologies) supplemented with 9% fetal bovine serum (v/v) (FBS lot K14133; Atlanta Biological), 1% penicillin–streptomycin (Life Technologies), and 1% GlutaMAX (Life Technologies). Cells were passaged using .25% trypsin-EDTA (Life Technologies) and maintained at 37°C. A mixture of GelMA and Saos-2 cells was created by adding 20 μL of a cell solution containing approximately 5000 cells/μL to 130 μL of GelMA prepolymer solution. Cell/GelMA solution was pipetted dropwise onto sterilized PCL, PCL/PLA, and PCL/HA scaffolds and subsequently crosslinked using a Hamamatsu LED Controller (Hamamatsu C11924-511; Hamamatsu Photonics K.K.). Viability of cells was analyzed using a Live/Dead assay on day 5. To evaluate cell viability, scaffolds were placed in media containing 1:2000 dilution calcein-AM (Life Technologies) and 1:500 dilution ethidium homodimer (Life Technologies) and incubated at 37°C for 1 h. After 1 h, scaffolds were cut into three equal pieces using single edge industrial razor blades and imaged using an epifluorescence microscope (Nikon, Eclipse E-400; Nikon Corporation). Raw .tiff images were taken for all samples and processed linearly for contrast and brightness using Adobe Photoshop CC 2015 (Adobe Systems, Inc.). Processed images were overlaid and false colored to create final live/dead images with green representing live cells and red representing dead cells. Brightfield images were not processed linearly for contrast and brightness. Images were also obtained using the Hirox KH-8700 digital microscope (Hirox-USA, Inc.).

Results

Three-dimensional printing using hybrid PCL spools

The overall process of manufacturing log-pile geometries using hybrid spools is depicted in Figure 1. To manufacture spools, a Randcastle microfilament extruder was used to combine base PCL material with either HA or PLA (Fig. 1A). Extruded filaments of an appropriate diameter of 1.75 ± .05 mm could then be wound and fed into a commercially available MakerBot Replicator 2 3D printer (Fig. 1B).

A range of process parameters used for 3D printing (Fig. 1C) were determined from the melting point and degradation point information for each spool, obtained from TGA. A set of temperatures was chosen within these ranges and optimized such that the spools could be fed into the machine in a reliable and repeatable manner. Temperature, which was the critical variable, was adjusted to allow easy printability (extrusion) using viscous melts.

For clarity, the ''diameter'' variable does not refer to the diameter of the spool, but rather to a number fed into the MakerBot Slicer program. This was necessary to compensate the pushing force on various spools. For PCL and PCL-HA spools, this variable was decreased from 1.75 mm (standard size) to 1.5 mm to force the extruder to push more filament out, effectively compensating for the differences in density from PLA (1.4 g/cm^3) to PCL (1.14 g/cm^3). Finalized log-pile prints were realized after optimizing the printing conditions (Fig. 1D).

Composition and mechanical properties of printed scaffolds

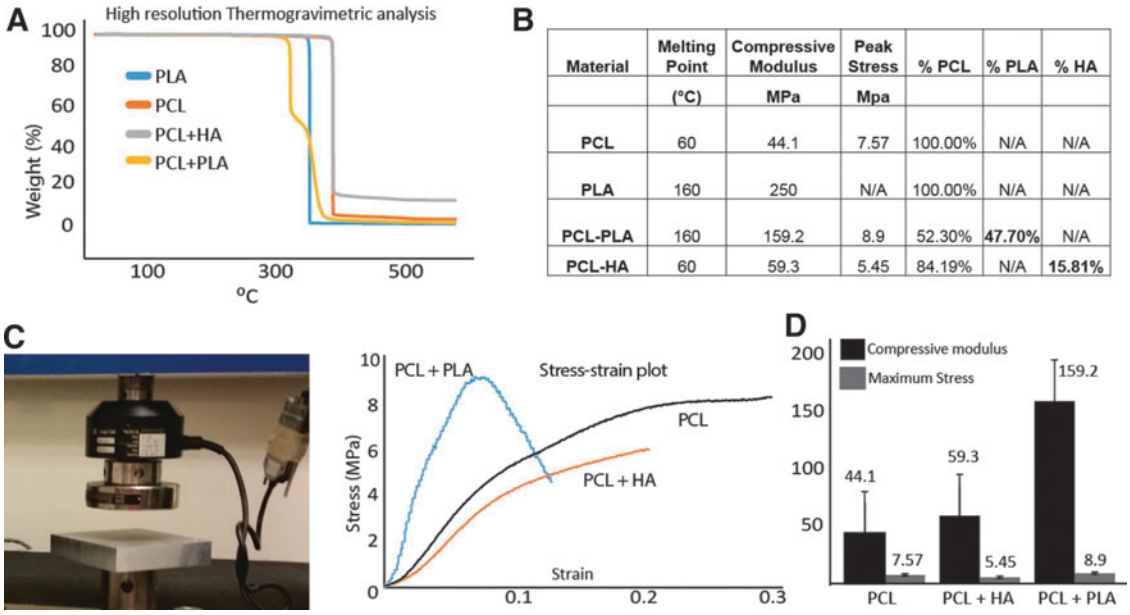
High-resolution TGA (Fig. 2A) was used to quantify the exact amount of filler (PLA or HA) in the PCL matrix. The sample was heated above its degradation point and the derivative of the heat of the furnace (the rate of heating) was measured as it related to the drop in weight of the test sample. This analysis was used primarily to demonstrate two distinct degradation points and a% composition of hybrid samples (Fig. 2A, B). Both PCL and PCL-HA had a degradation point of 383°C and the change weight between PCL and PCL-HA (15.81%) was representative of the weight of HA present in the PCL-HA spool, as inorganic HA does not degrade and remains on the balance after complete degradation of the PCL component. The actual amount of HA was found to be slightly lower after TGA than was present during the spool-making process (20%).

Similarly, the amount of PLA was found to be slightly lower after analysis. The mixture of PCL and PLA had two distinct degradation points (the first degradation point for PLA being at 325°C and the second point for PCL being at 355°C), which were used as markers for determining the percentage of each constituent in the combined spool (Fig. 2B). From the TGA, the exact amount of PLA in the PCL matrix was determined to be 47.7%, a minor deviation from the 50:50 ratio initially put into the extrusion hopper.

Mechanical properties for log-pile scaffolds were obtained from the stress–strain curves using a standard MTS machine (Fig. 2B–D). PCL-PLA, PCL-HA, and PCL had compressive moduli of 159.2 MPa, 59.3 MPa, and 44.3 MPa, respectively. The increase in moduli in the hybrid PCL-PLA spools was a result of the higher moduli of pure PLA (250 MPa) filler added to the PCL matrix.

Three-dimensional printing of complex porous geometries

PCL is a premier biopolymer used for bone tissue engineering applications and its ability to be readily augmented and printed in specific shapes via commercial 3D printers could lead to enhanced clinical solutions for bone defects. Custom scaffolds for bone repair are able to be printed from Cartesian data obtained from a CT scan, allowing the possibility of patient-specific support cages for bone tissue engineering (Fig. 3). The first geometry shown was created to have a log-pile lattice similar to the log-pile geometry



Material	Melting Point	Compressive Modulus	Peak Stress	% PCL	% PLA	% HA
	(°C)	MPa	Mpa			
PCL	60	44.1	7.57	100.00%	N/A	N/A
PLA	160	250	N/A	100.00%	N/A	N/A
PCL-PLA	160	159.2	8.9	52.30%	**47.70%**	N/A
PCL-HA	60	59.3	5.45	84.19%	N/A	**15.81%**

FIG. 2. **(A)** High-resolution TGA of polymer spools. **(B)** Results in tabular form showing the degradation points and percentages for each compound. **(C)** Compression testing apparatus to obtain mechanical properties of log-pile scaffolds. **(D)** Stress–strain plots and bar graphs of modulus and maximum stresses for each polymer spool. TGA, thermogravimetric analysis. Color images available online at www.liebertpub.com/3dp

described earlier, but modified with macroscales and boundaries derived from the .stl file of a human femur (Fig. 3A). A scaled femur model was placed into the mesh program and sliced to obtain a small cross section of the diaphysis. The Meshmixer program was then used to render the model into various different layers while keeping the same external geometry (Fig. 3B, red outline). The lattice structure was used to maximize the internal free space while minimizing the amount of material used to support reliable printing of the structure.

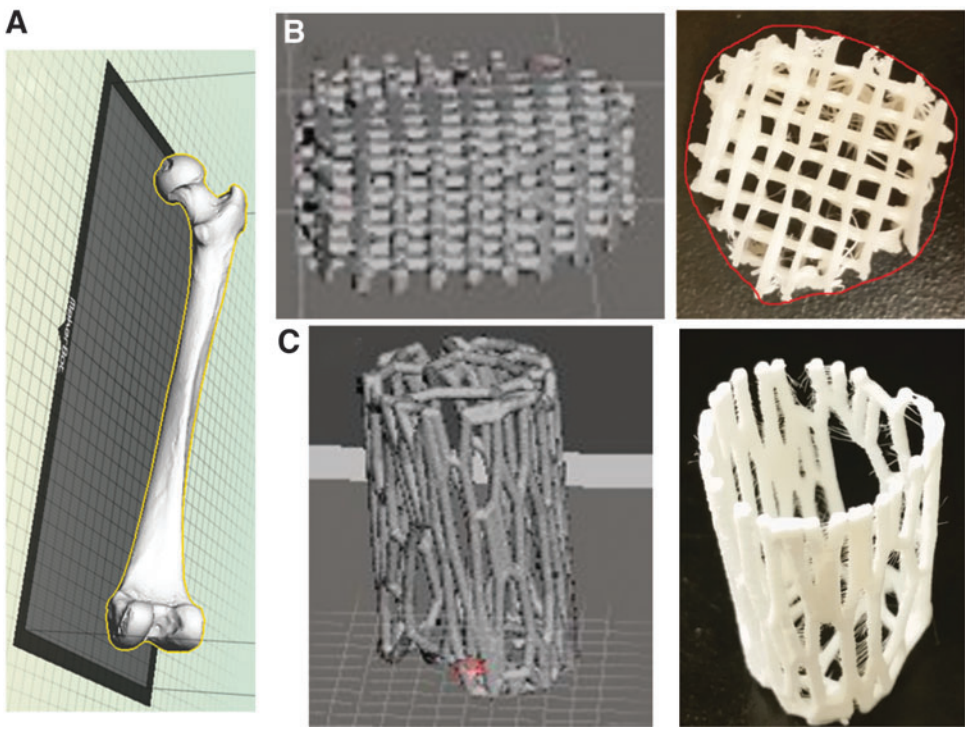


FIG. 3. **(A)** Cartesian data for a human femur were placed into the MakerBot Desktop. **(B)** A slice from the diaphysis of the femur was taken and Lattice meshed in Meshmixer. On the right is the 3D printed model in PCL. **(C)** Longer slice from the diaphysis was Voronoi meshed in Meshmixer to create a hollow cage-like representation of the outside of the femur. On the right is the 3D printed PCL model. Color images available online at www.liebertpub.com/3dp

To increase the internal free space, another type of scaffold was developed using Voronoi analysis, a solution that only uses the surface data in the .stl file (Fig. 3C). The Voronoi was made by reducing the number of triangles on the surface of the .stl file and subsequently using the Dual Edges function to give them all a tubular thickness and density. For each filament, the .stl's were uploaded into the MakerBot Desktop software and the settings were edited in the slicing program to obtain printing repeatability. The resulting hollow cages maintained the external geometry as derived from the CT scan, while still leaving space for the incorporation of biomaterials containing living cells.

Cell incorporation within log-pile scaffold

To visually model a process in which live cells could be contained within a 3D printed log-pile structure, a 6 mm^3 PCL log-pile scaffold was placed inside a rectangular chamber and subsequently covered with 7% GelMA containing 15% (v/v) MiO orange coloring (Kraft Foods, Inc.) (Fig. 4A). The GelMA was pipetted dropwise onto the PCL scaffolds and simultaneously solidified using UV light exposure. Sliced sections from the top, middle, and bottom areas were successfully able to show a thorough incorporation of GelMA within the interiors of the log-pile scaffold (Fig. 4B). As reported, the infusion process was facilitated by using a plastic column containing a small outlet port in the bottom of the chamber, thereby allowing an even flow-through of GelMA solution throughout the entire scaffold.

For cell encapsulation, a mixture of gelatin methacrylate (7% GelMA) and human osteosarcoma cells (Saos-2, 5000 cells/μL) was incorporated within a PCL-PLA log-pile structure in a similar manner. After day 5, the log-pile structure was incubated with calcein-AM and ethidium homodimer and imaged fluorescently for Live/Dead analysis (Fig. 4C). Live cells (green) were shown prevalently in the top, middle, and bottom sections of the sliced log-pile scaffold. Similar results were obtained for PCL and PCL-HA log-pile scaffolds (results not shown here).

Discussion

We choose PCL as our base material due to its extensive use in developing scaffolds for bone tissue engineering. PCL is a semicrystalline aliphatic polymer with suitable rheological properties, such as glass transition temperature and melting point, and is readily biodegradable by hydrolysis.[22] HA, while a major component of native bone that has been shown to promote bone mineralization, is difficult to process due to its brittle characteristics.[23,24]

One significant challenge in creating the composite spools was the difficulties in achieving filaments of consistent diameter (1.75 mm), a necessary requirement for several commercially available 3D printers. This aim was achieved by controlling the extruder pressure and barrel screw revolution, and by varying the ''suckback'' variable on MakerBot (Fig. 1C). The ''suckback'' is typically set at 1.3 mm at a velocity of 25 mm/s to break off each extrusion. Since PCL has a high specific heat, ''suckback'' was set to zero to avoid blocking the printer nozzle.

A second challenge that will need to be addressed in subsequent studies is the apparent loss of both HA and PLA in the spool manufacturing process (Fig. 2B). As previously mentioned, there was a slight decrease in the amount of HA and PLA measured in the composite PCL-HA and PCL-PLA spools compared to the starting materials placed into the hopper. While the exact cause of the loss is unknown, it is most likely attributed to the adhesion of HA and PLA to either the inner barrel of the extruder or any of the other various surfaces that they came in contact with throughout the manufacturing steps.

Natural bone is an organic/inorganic composite typically consisting of cortical and cancellous bone[25] with a compressive modulus of 131–224 MPa and 5–10 MPa, respectively.

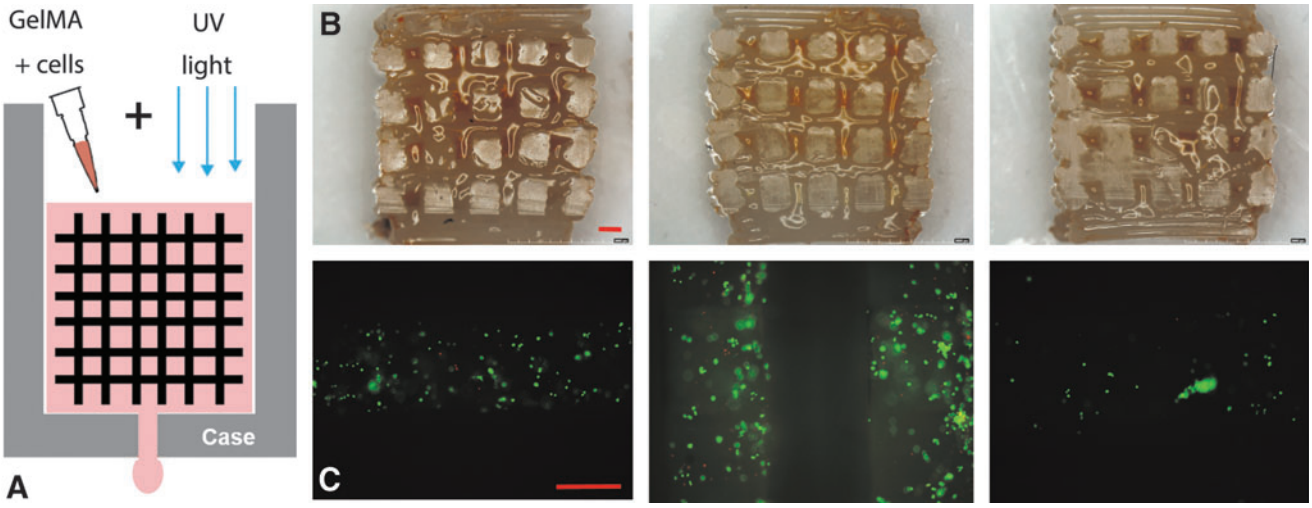


FIG. 4. **(A)** Schematic representation of the process used for incorporating living cells within printed scaffolds. Case was slightly larger than 6 mm^3 log-pile structure to allow for GelMA-cell solution perfusion. **(B)** PCL scaffold infused with 7% GelMA containing 15% (v/v) MiO *orange* coloring to show complete perfusion of GelMA throughout scaffold (*left* to *right*: PCL GelMA *top*, PCL GelMA *middle*, PCL GelMA *bottom*). **(C)** Live(*Green*)/Dead(*Red*) calcein-AM/ethidium homodimer pictures from PCL/PLA scaffold (*left* to *right*: PCL-PLA GelMA *top*, PCL-PLA GelMA *middle*, PCL-PLA GelMA *bottom*). GelMA, gelatin methacrylate. Color images available online at www.liebertpub.com/3dp

As reported here, the compressive results with the hybrid spools had moduli ranging 59–159 MPa, thereby falling well within the target zone required for bone tissue engineering. These results, in conjunction with the generation of a custom scaffold from CT scan data, bring credibility to the long-term goal of developing patient-specific support cages for bone tissue engineering.

Model scaffolds used for bone tissue engineering should mimic both the mechanical and chemical composition of natural bone. Since natural bone contains organic collagen fibers,[25] we decided to incorporate GelMA laden with living cells into the printed log-pile scaffolds. We chose GelMA, a denatured form of collagen, as the model hydrogel because it contains an abundance of biologically active cell-binding sites, has highly controllable mechanical properties, and provides increased transparency for imaging. Human osteosarcoma cells were chosen in this study due to the fact that they are a robust cell line commonly used in the initial stages of new bone defect models. By choosing osteosarcomas for our initial work, we were able to limit donor-dependent differences that would arise from primary cell lineages. In the future, we plan to use bone marrow-derived stem cells to obtain an understanding of how scaffold design parameters influence the cell number and their metabolic activities. Integration of the structural cage with cell-laden GelMA showed a construct compatible with the multifunctional nature of bone and showcased the potential for a tissue engineering model capable of improving the handling characteristics of soft hydrogel constructs under weight-bearing conditions. The open-frame design provided sufficient space for cell-laden hydrogels to support bone mineralization, remodeling, and integration with host tissues, while at the same time providing a stabilizing interface between engineered and host tissues.

Conclusion

Polymers, with their favorable processing properties, have been extensively used to develop 3D scaffolds for tissue engineering applications. 3D printing of tissue engineering scaffolds often requires customized instruments such as heated extruders and computer-controlled XYZ stages with precisely coordinated movements to lend themselves properly in the creation of biopolymer properties (viscosity, melting temperature, printing speed). This project developed hybrid spools with FDA-approved PCL polymer as the base material as well as hybrid spools readily compatible with commercial 3D printers such as the MakerBot. Porous geometries were printed using CT scan data with both internal log-pile lattice structures and external meshed structures. Finally, we demonstrated that living cells mixed in gelatin hydrogels could be incorporated within log-pile scaffolds with high cellular viability.

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Author Contribution

L.A. developed and characterized hybrid spools with log-pile and complex geometries. Cell experiments and characterization were carried out by S.W.S. P.S. conceived the project. All authors contributed to writing the article.

Author Disclosure Statement

The authors have no financial or nonfinancial relationships to disclose.

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