Radially and Axially Graded Multizonal Bone Graft Substitutes Targeting Critical-Sized Bone Defects from Polycaprolactone/Hydroxyapatite/Tricalcium Phosphate

Asli Ergun, Ph.D.,¹ Xiaojun Yu, Ph.D.,² Antonio Valdevit, Ph.D.,²
Arthur Ritter, Ph.D.,² and Dilhan M. Kalyon, Ph.D.^{1,2}

Repair and regeneration of critical sized defects via the utilization of polymeric bone graft substitutes are challenges. Here, we introduce radially and axially graded multizonal bone graft substitutes fabricated from polycaprolactone (PCL), and PCL biocomposites with osteoconductive particles, that is, hydroxyapatite (HA), and β -tricalcium phosphate (TCP). The novel bone graft substitutes should provide a greater degree of freedom to the orthopedic surgeon especially for repair of critically sized bone defects. The modulus of the graft substitute could be tailored in the axial direction upon the systematic variation of the HA/TCP concentration, while in the radial direction the bone graft substitute consisted of an outer layer with high stiffness, encapsulating a softer core with greater porosity. The biocompatibility of the bone graft substitutes was investigated using *in vitro* culturing of human bone marrow-derived stromal cells followed by the analysis of cell proliferation and differentiation rates. The characterization of the tissue constructs included the enzymatic alkaline phosphates (ALP) activity, micro-computed tomography imaging, and polymerase chain reaction analysis involving the expressions of bone markers, that is, Runx2, ALP, collagen type I, osteopontin, and osteocalcin, overall demonstrating the differentiation of bone marrow derived stem cells (BMSCs) via osteogenic lineage and formation of mineralized bone tissue.

Introduction

REPAIR OF LARGE segmental bone defects, that is, critical-sized defects, which occur due to blunt trauma, tumor resection surgeries, pathological degeneration, and congenital deformities, are challenges for orthopedic surgery.¹⁻⁴ Currently, about 5%–10% of procedures applied to repair critical-sized defects result in delayed unions or nonunions.¹⁻² Autografting is still the gold standard in clinical bone repair procedures.^{3,5-7} However, up to 30% of the autografting procedures have complications associated with the donor-site morbidity, limited tissue availability, varying quality, and longer hospital stays.⁶⁻⁸ Other alternatives, that is, allografts and xenografts, suffer from limited sources of supply, contamination risks, immunogenic incompatibility, and inability to incorporate with the host bone.^{3,7,8} The availability of synthetic bone grafts offers an alternative route in treatment of critical-sized defects.¹⁻⁸ Clinical trials have demonstrated the possibility of using metallic, ceramic, and polymeric bone graft substitutes.^{5,9-14} It is generally understood that the bone grafts need to be (i) biocompatible, (ii) preferably bioresorbable to prevent second surgery for removal after full recovery or due to late immunologic response, wear or dislodgement, (iii) mechanically adequate

and stable until functional bone tissue forms within the defected area, and (iv) most importantly osteoconductive and preferably osteoinductive.^{1,6-8,12}

A second approach, that is, bone tissue engineering, attempts to utilize porous polymeric scaffolds that are typically seeded with the patient's own stem cells. Tissue constructs are formed upon the proliferation and differentiation of the cells on the scaffold within a bioreactor and implanted to the defect site. Typical polymeric materials for such tissue engineering scaffolds are polyglycolide, polylactide, polycaprolactone (PCL), and their copolymers or their biocomposites with particles like hydroxyapatite (HA) and tricalcium phosphate (TCP). Other bioagents like growth factors can also be incorporated.^{6,7,9-14}

The designing and fabrication of bioresorbable polymeric bone graft substitutes and porous scaffolds for tissue engineering are challenged by the complex structural and compositional gradations found in human tissues.^{15,16} For example, it would be desirable for the bone graft substitutes used in the repair of critical-sized defects in long bones like femur and tibia to accommodate their changing porosities and moduli along their transverse and axial directions.^{17,18} Therefore, mimicking of such complex gradations found in native tissues can require correspondingly complex gradations in bone graft substitutes

Departments of ¹Chemical Engineering and Materials Science, and ²Chemistry, Chemical Biology and Biomedical Engineering, Stevens Institute of Technology, Hoboken, New Jersey.

and tissue engineering scaffolds that exhibit tailored three-dimensional distributions in composition, structure, and properties. However, past efforts to generate bone graft substitutes and scaffolds with such gradations were constrained by the available conventional scaffolding methodologies. Examples to recently developed structures involve the generation of multilayered scaffolds that were fabricated via electrospinning of separate meshes and pressing them under hydraulic pressure,¹⁹ electrospinning of contiguous nonwoven meshes with systematic changes in bioactive concentrations,^{20–22} and the use of a twin screw extrusion and spiral winding method to generate axially and radially graded scaffolds.^{23–24} Other methods of generating graded scaffolds include layer-by-layer casting, freeze-drying, phase separation, and rapid prototyping techniques, including fused deposition modelling, 3D printing, selective laser sintering, and stereolithography.^{15,16,25–31} However, additional methods that would allow the reproducible and industrially scalable grading of bone graft substitutes and tissue engineering scaffolds for a wide range of compositions, porosities, and mechanical properties are still needed.

Here we introduce a new class of radially and axially gradable porous structures from bioresorbable polymers and osteoconductive additives targeting bone graft substitute applications. They can also serve as scaffolds for bone tissue engineering. The fabrication of the graded structures was demonstrated here using PCL and biocomposites of PCL with HA and TCP. In the radial direction, the bone graft substitutes consist of an outer stiff layer of PCL incorporated with HA/TCP and a softer core layer from PCL. The outer layer exhibits lower porosity and smaller pore sizes, and the softer core layer exhibits higher porosity and larger pore sizes (inspired from cortical/cancellous bone). The interconnected nature of the porosities of the inner and outer layers should allow cell migration and exchange of nutrients and metabolic wastes in the radial direction during bone regeneration. Furthermore, the bioresorbable bone graft substitutes could also be graded in the axial direction. This is demonstrated here primarily by tailoring the concentration of the HA/TCP used in the fabrication of the outer layer as a function of axial distance to give rise to a relatively long bone graft substitute that is progressively stiffer in the axial direction.

The samples of the unitary radially and axially graded bone graft substitute were fabricated using a single continuous process, that is, coextrusion, which is ideally suited for fabrication of layered multizonal structures and which has rarely been applied in the field of bone regeneration and repair.³² The mechanical properties of the radially and axially graded coextruded bone graft substitutes were characterized using uniaxial compression loading and their biocompatibility was assessed via *in vitro* culturing of human bone marrow stem cells and the analyses of their proliferation and differentiation rates as a function of culturing time.

Materials and Methods

Materials

The materials of the study were similar to those used in an earlier study³³ and involved polycaprolactone (PCL) with a molecular weight of 70,000 g/mol (Scientific Polymer Products, Inc.), NaCl and polyethylene glycol (PEG, Mw = 35,000 g/mol from Sigma-Aldrich). The NaCl was ground and sieved to provide particles with two different particle

size ranges of 45–90 and 45–180 μm . The NaCl particles and PEG were used as primary and secondary porogens, respectively. Calcium phosphate ceramics, HA, and TCP (Sigma-Aldrich) were incorporated at a HA/TCP ratio of 20/80. This ratio is considered to be optimum, since earlier studies indicated that it provides osteogenic differentiation *in vitro* and faster bone generation *in vivo*.^{34–38}

Fabrication of functionally graded multizonal bone graft substitutes

The coextrusion process was based on the use of a twin screw extrusion process in conjunction with a coextrusion die designed to manufacture axially and radially graded multizonal bone graft substitutes. A 7.5-mm twin screw extruder with fully intermeshing and corotating screws was integrated with a coextrusion die to fabricate bone graft substitutes. A solventless PCL suspension consisting of 30 vol.% PCL, 20 vol.% PEG, and 50 vol.% NaCl with a particle size of 45–90 μm was fed into the feeding port of the twin screw extruder and incorporated with HA/TCP particles fed into a second feeding port of the twin screw extruder in a time-dependent manner (Fig. 1a). The time-dependent feeding of the HA/TCP particles (typically monotonically increasing and decreasing in progressive cycles) provided the facility to generate axial gradations in osteoconductivity and stiffness. This PCL suspension incorporated with HA/TCP in a time-dependent manner constituted the outer layer of the bone graft substitutes upon pressure-induced flow through the coextrusion die. On the other hand, the inner layer formulation (24 vol.% PCL, 36 vol.% PEG, and 40 vol.% porogen NaCl with particle size of 45–180 μm) was kept invariant with time and generated the inner layer of the bone graft substitutes upon passing through the coextrusion die (Fig. 1a). The rotational speed of the two screws was maintained at 140 rpm and the temperatures of the barrel and the die of the extruder were kept within the 71°C–74°C range. The absence of a solvent is an asset of the process.

Characterization of bone graft substitutes

The continuously coextruded functionally graded bone graft substitute specimens with a cross-sectional area of about 6 mm by 10 mm, were sliced at regular intervals of 2–2.5 mm to allow the analysis of different segments along their axial lengths (Fig. 1b). They were designated as S0 to S5, indicative of the first and sixth segments over a length of 100 mm. The concentration of the HA/TCP was increased from 0% to 24% by weight at the outer layer of the bone graft substitute over this length of 100 mm. Thus, S0 contained 0% HA/TCP, whereas segment S5 contained 24% HA/TCP with a linear increase of the HA/TCP concentration in between these two segments (Fig. 1c). Upon extrusion and slicing, the bone graft substitute specimens were immersed in distilled water until all of the porogens, that is, PEG and salt could be removed (the leaching/extraction process was monitored via successive weight measurements until only PCL remained).

The distribution of the HA/TCP concentration in the outer layer of the bone graft substitutes was characterized using thermogravimetric analysis (Q100, TA Instruments) during which the specimens were subjected to heating from 25°C to 600°C at a rate of 200°C/min and kept at 600°C for a duration of 3 min. Under these conditions only the HA and TCP

FIG. 1. The fabrication of bone graft substitutes, which are graded in both radial and axial directions, that is, multizonal cage and core structures with greater porosity in the core with the concentration of osteoconductive particles, hydroxyapatite (HA)/tricalcium phosphate (TCP), of the cage layer increasing with axial distance (a). The insets show the actual extrudates that are fabricated continuously and cut to different lengths (b); demonstration of axial grading capability, that is, increasing concentrations of HA/TCP in the axial direction (c); modulus change of the bone graft substitute in the axial direction, corresponding to the axially increasing concentration of HA/TCP (d). Scanning electron microscope (SEM) micrographs of the first segment (S0) and last segment (S5) of the functionally graded bone graft substitutes, which show the porosity of the cage layer before cell seeding (e). PCL, polycaprolactone.

particles remained, that is, the pyrolysis of the PCL was completed around 350°C.

An AURIGA®-CrossBeam® Workstation (Carl Zeiss NTS) scanning electron microscope (SEM) was employed for SEM imaging of the tissue constructs at 5 kV. All samples were mounted onto Al stubs and coated with Au for 60 s using an SPI Module sputter coater (Structure Probe, Inc.). Energy dispersive spectroscopy (EDX) was conducted on harvested tissue constructs. The EDX conditions involved scanning for Ca and P for a duration of 150 s at 10 kV.

Unconstrained uniaxial compression tests ($n=6$) were performed on different segments of functionally graded bone graft substitutes using a Bose ELF 3300 (Eden Prairie) materials testing machine. The crosshead speed was 1 mm/min and the tests were conducted while the bone graft substitutes were maintained in phosphate-buffered saline (PBS) at room temperature. The Young's modulus (E), yield stress (σ_y), ultimate stress (σ_u), toughness, strain at break (ϵ_{break}), and strain at yield (ϵ_{yield}) values were determined from the compressive elastic region of the resulting stress-strain

curves of the different segments following procedures reported earlier.³³

In vitro cell culturing of human bone marrow stem cells on functionally graded bone graft substitutes

For the assessment of the biocompatibility of the bone graft substitutes, specimens pertaining to different segments were randomly selected. They were sterilized by being immersed in 70% ethanol for 1 h, rinsed with PBS for several times, and left under UV light for 30 min in PBS. The specimens were then washed several times with PBS and immersed in a growth medium as defined below for an hour before cell seeding.

Human bone marrow stem cells (Poietics) available from Lonza Walkersville, Inc. were used for *in vitro* culturing.³³ Both sides of the bone graft substitutes were seeded at 2.0×10^4 cells/scaffold (1.0×10^4 cells/side). One microliter of the growth medium was added into each well to immerse the specimens completely to be followed by culturing in an

incubator at 37°C and 5% CO₂. The growth medium used during the first 4 days of culturing contained a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium–low glucose, supplemented with an antibiotic solution (1% penicillin/streptomycin; Sigma-Aldrich) and 10% fetal bovine serum.³³ On day 4, the growth medium was replaced with the osteogenic differentiation medium, that is, the growth medium supplemented with 50 µg/mL ascorbic acid, 10 mmol β-glycerophosphate, and 10 nmol dexamethasone. The media were changed every 2 days, and the constructs were flipped during every medium change.³³ At days 7, 14, 21, and 28 samples of the tissue constructs were harvested and characterized for cell proliferation, differentiation, and morphological analysis.

Cell viability and proliferation

The cell viability and proliferation rates were characterized using PicoGreen[®] Assay (Invitrogen-Molecular Probes) following the manufacturer instructions and the cell number was determined using a calibration curve that was established by DNA isolated from using a known number of cells counted by a hemacytometer.³³ The results were reported as number of cells/scaffolds.

Alkaline phosphatase activity

The development of the osteoblastic phenotype from stem cells was assessed by measuring alkaline phosphatase (ALP) activity using a colorimetric method based on the conversion of p-nitrophenyl phosphate into p-nitrophenol in the presence of ALP following procedures reported earlier.³³ The protein amount was determined through a calibration curve that was established using a list of known standard bovine serum albumin (BSA) (Sigma-Aldrich) solutions. The results for ALP activity assay of samples harvested on days 7, 14, 21, and 28 were reported as nmol of ALP/mg of total protein.

Reverse transcription–polymerase chain reaction

Tissue constructs (*n*=3) at 7, 14, 21, and 28 days were transferred into 2-mL plastic tubes, and 1.5 mL Trizol (Sigma-Aldrich) reagent was added to isolate total RNA from the constructs using the manufacturer's instructions and following procedures reported earlier.³³ The oligonucleotide primer sets used for polymerase chain reaction (PCR) analysis and marker-specific annealing temperatures are given in Table 1.³⁹ The PCR products were run through a 1.5% agarose gel electrophoresis unit and visualized by

ethidium bromide staining. For quantification of the expressions, the band intensities were analyzed by NIS-Elements software (Nikon) and the expression values of the markers were normalized to the expression of the house-keeping gene, GAPDH.

Microcomputed tomography evaluation of the tissue constructs

The whole samples of the tissue constructs were subjected to microcomputed tomography (µ-CT) analysis using a Scanco mCT 35 (Scanco Medical) system following procedures that were reported earlier.³³ The Scanco µCT software (HP, DECwindows Motif 1.6) was used for 3D reconstruction and viewing of images using a global threshold of 59 mg HA/cm.³

Statistical analysis

Statistical analysis was performed using SigmaPlot 11.0 software package. Normality assumption and equal variance were verified by setting the *p*-value to 0.05 using the Shapiro–Wilks's and Levene Median tests, respectively. Statistical significance for the change of HA/TCP concentrations as well as mechanical properties along the axial direction of the bone graft substitutes were investigated using one-way analysis of variance (ANOVA) followed by the Tukey's *post hoc* test. Two-way ANOVA followed by the Bonferroni *post hoc* procedure were then used to test for significance in cases of multiple comparisons, including cell differentiation rates, ALP activity, and marker expression levels. All data are provided as 95% confidence intervals determined according to Student's-*t*-distribution.

Results

Fabrication of radially and axially graded bone graft substitutes and their characterization

The radially and axially graded multizonal bone graft substitutes, fabricated using coextrusion, are shown in Figure 1b. The pore sizes were 55–200 µm and 1–110 µm for the inner and outer layers, respectively. The coextrusion process with time-dependent changes in the feeding rates of the ingredients enabled the generation of composition and porosity variations in the radial direction and compositional distributions in the axial direction (with also minor changes in porosity in the outer layer of the bone graft substitute) within the body of a unitary bone graft substitute. Segments from the coextruded strand were collected systematically

TABLE 1. REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION PRIMERS AND THEIR ANNEALING TEMPERATURES

	Forward primer	Reverse primer	Size (bp)	<i>T</i> _{annealing} (°C)
Runx2	5'-CACCATGTCAGCAAACTTCTT-3'	5'-TCACGTCGCTCATTTTGC-3'	96	55
Alkaline phosphatase	5'-CCATCCTGTATGGCAATGG-3'	5'-CGCCTGGTAGTTGTTGTGAG-3'	93	57
Collagen I	5'-CAAGAGTGGTGATCGTGGTG-3'	5'-GCCTGTCTCACCTTGTCA-3'	118	57
Osteopontin	5'-GAGGGCTTGGTTGTCAGC-3'	5'-CAATTCTCATGGTAGTGAGTTTCC-3'	129	59
Osteocalcin	5'-TGAGAGCCCTCACACTCCTC-3'	5'-ACCTTTGCTGGACTCTGCAC-3'	97	59
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'	65	59

TABLE 2. UNIAXIAL COMPRESSIVE PROPERTIES OF THE FUNCTIONALLY GRADED MULTIZONAL BONE GRAFT SUBSTITUTES

	E MPa	σ_Y MPa	σ_U MPa	Toughness N/m ²	ϵ_{yield} (mm/mm)	ϵ_{break} (mm/mm)
S0	1.40±0.61	1.09±0.08	1.76±0.09	1.21±0.10	1.81±0.37	2.38±0.54
S1	2.56±1.02	1.34±0.05	2.02±0.12	0.92±0.26	1.65±0.31	2.18±0.41
S2	4.84±1.17	1.37±0.07	2.06±0.04	0.96±0.35	1.49±0.51	2.05±0.53
S3	5.15±2.07	1.44±0.05	2.25±0.12	1.60±0.59	1.35±0.57	2.01±0.56
S4	5.45±1.63	1.47±0.04	2.26±0.09	1.62±0.52	1.26±0.29	1.92±0.38
S5	5.47±1.55	1.50±0.03	2.40±0.09	1.66±0.48	1.19±0.35	1.83±0.41

S0, first segment, ... , S5, last segment.

and the TGA results confirmed the gradual increase in the HA/TCP concentration from 0% to 24% by weight in the outer layer (Fig. 1c). In the radial direction, the porosity values of the inner layer of the bone graft substitutes were kept constant at 80.0%±0.1%, while the porosity of the outer layer was decreased from 75.4%±0.7% to 70.3±1.7 for going from segment 0 to 5.

The ability to alter the concentration distributions of the HA/TCP as a function of axial distance allowed the tailoring of the mechanical properties of the bone graft substitutes in the axial direction. Thus, overall, the mechanical properties were different for the outer and inner layers and also changed along the axial length of the bone graft substitutes, that is, the compressive modulus of the inner layer was 0.95±0.37 MPa and the modulus of the outer layer could be altered between to 1.40±0.61–5.47±1.55 MPa along the length of the bone graft substitute (Fig. 1d). The elastic modulus, the stress values at yield and failure and the toughness values also increased with the gradual increase of the HA/TCP concentration in the outer layer (Table 2). On the other hand, the strain at yield and break values decreased with the increase of the HA/TCP concentration at the outer layer. Thus, overall, the location dependence of the compressive properties (Table 2) attest to the demonstration of the ability to alter the mechanical properties in a systematic manner along the axial direction of the bone graft substitutes.

Cell–scaffold interactions

The biocompatibility of the functionally graded multizonal bone graft substitutes was evaluated via seeding with human bone marrow derived stem cells (BMSCs) and *in vitro* culturing for a total duration of 4 weeks. Acceptable bone graft substitutes for bone regeneration need to support the proliferation and differentiation of cells, which involves cell attachment and proliferation, extracellular matrix (ECM) maturation, and ECM mineralization.^{40,41} The results showed a monotonic increase of the number of BMSCs on functionally graded bone graft substitutes with incubation time during 4 weeks (Fig. 2). When compared to bone graft substitute samples without any HA/TCP, the gradual increase in the concentrations of HA/TCP promoted greater rate of cell proliferation and significantly increased the cell number as of the completion of the first week of culturing ($p < 0.01$). The cell attachment and proliferation on the bone graft substitutes were also documented via SEM imaging for the tissue constructs harvested after 28 days as shown in Figure 3. The SEM micrographs reveal the surface coverage of the bone graft substitutes by cells after 4 weeks of culturing.

Expressions of ALP were used for the assessment of BMSC differentiation of the tissue constructs. The tissue constructs displayed a statistically significant monotonic increase of the ALP expression (Fig. 4) up to three to 4 weeks ($p < 0.05$) consistent with the earlier *in vitro* studies on differentiation of stem cells on PCL bone graft substitutes incorporated with HA and TCP.^{33,42–44} Tissue constructs harvested at day 21 exhibited a significant difference in ALP expression levels ($p < 0.05$) where the gradual increase in the HA/TCP concentration promoted higher ALP activity. As a consequence of mineralization, the ALP activity detected from the segments 4 and 5, decreased between the third and fourth weeks of culture, consistent with earlier studies.^{33,40}

Gene expression of Runx2, ALP, collagen type I, osteopontin, and osteocalcin evaluated by reverse transcription–PCR

The expressions of Runx2, ALP, collagen type I, osteopontin, and osteocalcin are reported in Figure 5 and confirmed the osteogenic differentiation of the BMSCs toward bone tissue and formation of a mineralized matrix. The Runx2 is a master regulator in osteogenic differentiation and its expression upregulates the osteoblast-specific genes, including collagen type I, osteopontin, and osteocalcin, and promotes ALP

FIG. 2. Cell proliferation rates of bone marrow derived stem cells (BMSCs) on axially graded segments of bone graft substitutes containing different concentrations of HA/TCP. BMSCs were cultured for 4 weeks *in vitro* and the cell proliferation rates were determined at days 1, 7, 14, 21, and 28 of culturing periods via PicoGreen[®] Assay. Statistical significance $p < 0.001$ is denoted by ***.

FIG. 3. SEM micrographs of tissue constructs after 4 weeks of culturing. The micrographs show the proliferation and spreading of BMSCs after 28 days in culture. The circles point to the cells present on the bone graft substitutes.

activity and mineralization.^{45,46} The Runx2 expression (Fig. 5a) was significantly high at the fourth week of culturing when compared to first 2 weeks of culturing ($p < 0.5$). Even though the increase in the concentration of HA/TCP increased the expression levels between third and fourth weeks of culturing, the difference was not statistically significant.

ALP mRNA expression levels (Fig. 5b) increased significantly between the second and third weeks of culturing ($p < 0.05$). Tissue constructs harvested at day 21 exhibited a significant difference in ALP mRNA expression levels between segments 0 and 5 ($p < 0.05$) where increasing the HA/TCP concentration promoted higher ALP activity. This is consistent with the enzymatic ALP expression pattern de-

termined on tissue constructs harvested on day 21 (Fig. 4). Collagen type I is the main type of collagen found in the bone matrix and is a marker of osteoblastic differentiation and bone ECM formation.^{47,48} The expression level of collagen type I significantly increased during 4 weeks of culturing (Fig. 5c) and the increase was statistically significant between the third and fourth weeks of culturing, indicative of the formation of bone ECM. Furthermore, the collagen type I expression levels of the tissue constructs harvested at days 21 and 28 were significantly higher for the segments with the higher HA/TCP concentration ($p < 0.05$).

Although an increase of the osteopontin expressions with culturing time was suggested by the data, some of the findings were not statistically significant (Fig. 5d). On the other hand, the monotonic increase of the osteocalcin expression with culturing time (Fig. 5e) was statistically significant and was indicative of osteoblast maturation and matrix mineralization upon the onset of nodule formation ($p < 0.01$). Additionally, after 3 weeks of culturing, the tissue constructs of segment 4 and 5 with the higher HA/TCP concentrations showed significantly higher osteocalcin expressions in comparison to segments 0, 1, and 2, which contain lower concentrations of HA/TCP ($p < 0.05$).

μ -CT evaluation of the tissue constructs upon 4 weeks of in vitro culturing

The heavy mineralization revealed by the observed significant increase of the osteocalcin expression was further confirmed with the μ -CT imaging results (Fig. 6). Mineralization is indicated by the change in color from blue to light gray. The μ -CT analysis revealed significant matrix mineralization especially at the outer layer. The gradual increase in the HA/TCP concentration promoted the mineralization and bone formation, which is indicated by the greater degree of mineralization with time achieved by segments with initially greater HA/TCP concentrations. The EDX analysis (Fig. 7) of the elemental Ca and P distributions verified the calcification

FIG. 4. Alkaline phosphatase (ALP) activity versus culture time of BMSCs from tissue constructs harvested following culturing periods of 7, 14, 21, and 28 days (* denotes statistical significance with $p < 0.05$). The ALP activity increased for all segments during the first 21 days with the greatest increase observed for segments containing the greatest concentrations of HA/TCP, that is, segments 4 and 5.

FIG. 5. The expressions of bone tissue-specific markers, including Runx2 (a), ALP (b), collagen type I (c), osteopontin (d), and osteocalcin (e) were evaluated during the *in vitro* culturing period of up to 28 days to assess osteogenic differentiation of BMSCs (*, ** denote statistical significance with $p < 0.05$, $p < 0.01$, respectively).

of the ECM. The intensities of the peaks associated with the elemental Ca and P increased with the increase of the initial HA/TCP concentration.

Discussion

In massive traumatic bone losses, the defects exceeding a critical size (typically greater than 3 cm for the forearm, 5 cm for the femur and tibia, and 6 cm for the humerus) cannot be healed via mechanical fixation and a graft is necessary to fill the gap of the defect.⁶ Conventionally, the implants utilized in repair of critical-sized bone defects have homogeneous structures and material properties. However, it can be considered that the success rate of repair/regeneration of critical-sized defects would increase significantly if the structure and hence, the properties of bone graft substitutes can be tailored and optimized in a three-dimensional manner to satisfy the specific requirements of the defect and the implantation site.¹⁶ Here we have introduced bone graft substitutes that could be graded in composition and structure in

a three-dimensional manner to provide additional degrees of freedom to guide cell/material interactions, including cell migration, vascular infiltration, passage of blood and nutrients, and removal of metabolic byproducts, and mechanical and degradation properties. The demonstration was carried out using bioresorbable PCL and its biocomposites with HA and TCP. However, it should be recognized that other types of polymers or other osteoconductive additives or other bioactives could also have been incorporated in a graded manner using similar methodologies.

Both structural and compositional gradations could be introduced concomitantly into the unitary multizonal bone graft substitutes. Typically, at least two processes in tandem are necessary to generate axially graded structures to serve as graft substitutes or scaffolds for bone tissue engineering. For example, Eriskin *et al.* have developed a hybrid technology, which marries the twin-screw extrusion process with electrospinning. This hybrid process was used to fabricate functionally graded nanoparticle-incorporated nanoporous meshes of PCL- β -TCP for cartilage-bone interface

FIG. 6. The whole tissue constructs of bone graft substitutes harvested at the end of the culturing period were analyzed by μ -CT scan. The images show the new bone tissue formation on the bone graft substitutes cultured for 28 days revealed by the change of color from blue to light gray. The increasing HA/TCP concentration supported bone tissue formation on the bone graft substitutes. Color images available online at www.liebertpub.com/tea

regeneration.^{20–22} In a second example, Ozkan *et al.*, have integrated the twin-screw extrusion process with the spiral winding process to construct tubular porous radially graded PCL- β -TCP scaffolds for bone regeneration studies.^{23–24} The coextrusion methods introduced here are complementary to these earlier methodologies and would significantly expand the geometries, structures, and functionalities of polymeric bone graft substitutes and scaffolds.

The bone graft substitutes demonstrated here consisted of a relatively stiff outer layer (uniaxial compressive moduli in the range of 1.4–5.5 MPa) and a softer core layer with greater porosity (uniaxial compressive modulus of around 0.95 ± 0.37 MPa). This design is akin to the cortical/cancellous bone structure in which the modulus of the cortical bone (12–20 GPa) is significantly greater than that of the cancellous bone (1.7–1624 MPa) and highly dependent on the anatomical

FIG. 7. The energy dispersive spectroscopy spectra for elemental Ca and P of the tissue constructs harvested upon 4 weeks of *in vitro* culturing. The SEM micrographs show the locations that are scanned. The whole area shown on the micrographs were scanned to evaluate ECM mineralization after 28 days of *in vitro* culturing. The functionally graded bone graft substitutes demonstrated more distinctive elemental Ca and P peaks with increasing initial HA/TCP concentration, that is indicative of enhanced mineralization. Color images available online at www.liebertpub.com/tea

location.^{8,49} Furthermore, the compressive modulus of the bone graft substitute could be altered systematically in the axial direction (from 1.40 ± 0.61 to 5.47 ± 1.55 MPa) also by utilizing an axial location-dependent concentration distribution of the osteoconductive agents, HA and TCP particles.

Applications of this bone graft substitute/scaffolding technology provide several unique advantages in the field of orthopedics. First of all, the coextrusion process permits fabrication of the bone graft substitute into a variety of geometric shapes. Perhaps, more importantly is the customization of the modulus of the resulting bone graft substitute in both the axial and radial directions. This unique property will permit use of the device as a bone graft in cases of long bone resection as in tumor removal procedures. In these cases, the current practice involves insertion of autograft in conjunction with external fixation. Such a condition can be susceptible to both postoperative infections as well as stress shielding. Use of the bone graft substitute demonstrated here will permit dynamization via a reduced modulus inner core combined with a longitudinal modulus variation in an effort to provide functional support, and yet remain ductile to promote bone remodeling.⁵⁰

Dynamization has been shown to promote early bone healing and the local movement at the site can be correlated to the loading within the bone/fracture site complex.⁵¹ Further, dynamization has had reports of decreased infection with respect to the pins utilized for external fixation.⁵² With customizable moduli, one may design and extrude spacers with compressive properties suitable for bone spacers, intervertebral spaces for degenerative spine that can be used in conjunction with either internal or external instrumentation. As well, the modulus can be tailored to the anticipated loading based on the patient size, thereby allowing reducing the cases of stress shielding for those smaller patients, while safeguarding against premature subsidence in larger patients. Thus, the flexibility associated with the fabrication of our graded bone graft substitutes can provide unique advantages in tailoring of geometries and properties according to the needs of the surgical procedure and the patient.

Furthermore, studies have shown that the matrix mechanical properties can be used as physical cues in the differentiation of stem cells into different lineages.^{53,54} It is recently revealed that the biological outcomes resulting from tissue engineering approaches depend on the physical properties of the scaffolding materials, such as topography, geometry, porosity, and stiffness. Such property variations along the length of the scaffolding materials were shown to direct the organization and the proliferation, migration and differentiation rates of stem cells akin to providing chemistry- or biochemistry-based signals varying along the length of the tissue engineering scaffolds.^{53,54} For example, Chatterjee *et al.* demonstrated the increase of mineralized tissue formation by osteoblasts along the length of scaffolds concomitant with the increasing modulus values of the hydrogels.⁵⁴ Thus, the ability of our coextrusion method to alter systematically the modulus of the bone graft substitute or the scaffold in tissue engineering applications in both the radial and axial directions, that is demonstrated here, should provide additional degrees of freedom for the selective differentiation of stem cells in a three-dimensional manner.

It was important to evaluate the biocompatibility of the bone graft substitutes, since they should enable the migration and expansion of indigenous osteoprogenitor cells within the defect site during healing.⁵⁵ Furthermore, it was of interest to determine if the differentiation rates of the stem cells would be affected by the changes in the compositions and mechanical properties along the radial and axial directions of the bone graft substitutes. This was assessed by seeding BMSCs onto the bone graft substitutes and their culturing for 4 weeks within first growth media and then differentiation media.

The conversion of BMSCs to bone tissue would involve cell attachment and proliferation, osteogenic differentiation indicated by a sequence of bone tissue-specific gene expressions and cell activities,⁵⁶ ECM formation, and mineralization.^{40,41} The commitment of progenitor cells into osteogenic lineage begins with activation of osteogenic master genes, that is, *Runx2*.⁵⁷ When cells are committed to the differentiation stage, several matrix proteins, including collagen type I and ALP, are upregulated to form bone ECM.^{56,57} The increase of enzymatic activity of ALP would be associated with increasing osteoblastic phenotype *in vitro*.⁵⁴ Expression of early markers of osteogenic differentiation would be followed by osteocalcin and osteopontin production and the cells would reach the mineralization phase with mineral nodule formation.^{56,57} Osteopontin, which contains phosphorylated sialoprotein would be present within the mineralized bone matrix and would be localized at sites of bone formation and resorption.⁵⁵ Osteocalcin, that is, a non-collagenous protein that contains three γ -carboxyglutamic acid residues, which allow it to bind to calcium, would facilitate ossification.⁵⁸

The harvesting of the tissue construct specimens as a function of culturing time and their subsequent PCR and ALP and μ -CT analyses indicated that these sequences of osteogenic differentiation events and their marker expressions indeed took place during the culturing of the BMSCs on our bone graft substitutes. The increasing enzymatic activity and expression of ALP mRNA suggested the onset of osteogenic differentiation and preparation of the ECM for mineral deposition.^{40,55,56} Furthermore, the cells continued to produce collagen type I during the entire culturing period, which indicated the bone ECM formation. The production of collagen type I slowed down between third and fourth weeks of culturing. Increasing osteopontin and osteocalcin expression with culture time suggested the osteoblast maturation, matrix mineralization via nodule formation. Furthermore, the presence of higher concentrations of HA/TCP promoted higher proliferation rates of BMSCs and greater enzymatic ALP activity as well as increased expression levels of the ALP, collagen type I, and osteocalcin markers. Overall, the observed culturing time-dependent increases of the BMSC proliferation rates, enzymatic ALP expressions, and the expressions of bone markers, suggest the differentiation of the BMSCs on the bone graft substitutes through osteogenic lineage and formation of mineralized bone tissue (also confirmed via μ -CT). The suggested differentiation of the stem cells and the observed expression of osteoblast phenotype markers are consistent with the expected functional activities associated with the progressive formation of bone tissue⁴⁰ and point to the biocompatibility of our bone graft substitutes that were fabricated in a radially and axially graded manner.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dilhan M. Kalyon, Ph.D.

Stevens Institute of Technology
Department of Chemistry, Chemical Biology and Biomedical
Engineering or Department of Chemical
Engineering and Material Science
Castle Point Street
Hoboken, NJ 07030

E-mail: dkalyon@stevens.edu

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