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Fabrication of Trabecular Bone-Templated Tissue-Engineered Constructs by 3D Inkjet Printing

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TOC image

A tandem μ CT/inkjet 3D printing process has been developed to fabricate tissue-engineered bone constructs (TEBCs) from human trabecular bone templates. TEBC morphometric properties, trabecular interconnectivity, surface roughness, and mechanical properties resemble those of human bone. Human mesenchymal stem cells cultured on the TEBCs exhibit significantly different metabolic activity, osteogenic differentiation, and mineralization depending on the anatomic site.

Keywords

additive manufacturing; bone tissue engineering; *in vitro* model

The development of three-dimensional (3D) *in vitro* drug screening tools and disease models offers the potential to significantly advance fundamental understanding of disease, drug discovery, and patient-specific precision medicine.^[1–5] Recent advances in 3D fabrication techniques have enabled studies on how properties such as matrix rigidity, surface chemistry, porosity, and curvature contribute to molecular mechanisms involved in disease progression.^[6–9] Additive manufacturing (AM), also known as 3D printing or rapid prototyping, offers the advantage of creating 3D objects with precisely controlled geometries and topological properties for multiple tissue types, including musculoskeletal tissues such as cartilage and bone. Properties of the bone microenvironment, including matrix rigidity^[7], surface curvature, and pore geometry^[6,10], regulate bone cell activity and the bone remodeling process that can be disrupted in a diseased state, which highlights the need to recapitulate the physicochemical, mechanical, and morphometric properties of bone in tissue-engineered models. In this work, we hypothesized that the anatomic site-specific architecture of trabecular bone regulates osteoblast differentiation and mineralization. To test this hypothesis, we fabricated human bone-templated 3D constructs via a new micro-computed tomography (μ CT)/3D inkjet printing process. We show that this process reproducibly fabricates tissue-engineered bone constructs (TEBCs) that recapitulate the anatomic site-specific morphometric and mechanical properties of trabecular bone. A significant correlation was observed between the Structure Model Index (SMI, a morphometric parameter related to surface curvature) and the degree of mineralization of human

mesenchymal stem cells (hMSCs), with more concave surfaces promoting more extensive osteoblast differentiation and mineralization compared to predominately convex surfaces.

Recent studies have combined CT imaging with AM to fabricate patient-specific bone scaffolds at the anatomic scale. These studies highlight the potential of AM for fabricating anatomically-scaled bone scaffolds with tissue-matched mechanical properties that incorporate multiple cell types and integrate with host tissue in animal models.^[11–14] However, anatomically-scaled 3D-printed scaffolds typically lack sufficient resolution to recapitulate the <100 micron-scale trabecular architecture essential for investigating the cellular response to the morphometric properties of bone. A limited number of recent studies utilizing 3D-printed scaffolds with controlled microstructure have reported that matrix rigidity, pore size, and pore shape regulate angiogenesis and osteogenesis *in vitro*.^[7,15–19] However, the effects of the anatomic site-specific morphometric properties of trabecular bone on osteoblast activity have not been investigated. Bone-templated scaffolds must also be fabricated from biomaterials that support bone cell function, which is challenging due to the limited number of substrates that can be printed.^[20,21] In this study, we used a templating approach in which 3D-printed wax molds were filled with a reactive, flowable, and settable poly(ester urethane)-nanocrystalline hydroxyapatite (PUR-nHA) hybrid polymer with bone-like strength. We have shown that this organic-inorganic hybrid polymer promotes osteoblast differentiation and mineralization, supports osteoclast-mediated resorption, and remodels to form new bone *in vivo*.^[22,23]

TEBCs were fabricated by a process utilizing μ CT technology in tandem with a Solidscape[®] 3Z Studio inkjet 3D printer (Fig. 1A). Human cadaver samples of the femoral head (FH), proximal tibia (PT), and vertebral body (VB) were scanned by μ CT and the images inverted and converted to the STL file format compatible with the 3D printer. These three anatomic sites exhibit different morphometric properties, thereby enabling testing of the hypothesis that the surface curvature of trabecular bone regulates hMSC proliferation, differentiation, and mineralization. Specific sites within the cadaver specimens were chosen such that the morphometric parameters were within the range of previously established literature values for each anatomic site.^[24] A reactive polyurethane-hydroxyapatite (PUR-nHA) hybrid composite composed of lysine methyl ester diisocyanate (LDI), nanocrystalline hydroxyapatite (nHA), polycaprolactone triol (PCL 300), and iron catalyst (5% iron acetylacetonate (FeAA) in ϵ -caprolactone), was poured into and drawn through the 3D wax templates under vacuum. The resulting hybrid polymer consisted of 52 wt% nHA, which falls in the range of the hydroxyapatite component of bone (50–70 wt%).^[25] After curing overnight at 50°C, the wax was leached from the TEBCs by immersion in acetone and the resulting PUR-nHA scaffolds scanned by μ CT to assess their similarity to the host bone template (Fig. 1B–C). The bone morphometric parameters Bone Volume/Total Volume (BV/TV), Trabecular Separation (Tb.Sp.), Trabecular Number (Tb.N.), and Structure Model Index (SMI) were calculated for the host bone templates and the printed TEBCs (Fig. 1C). Structure model index is a measure of the relative prevalence of plates (SMI = 0) versus rods (SMI = 3)^[26] and is thus a measure of surface convexity, since regions of bone containing enclosed cavities can have negative SMI values. Bone morphometric parameters for the host bone and TEBCs followed similar trends. Furthermore, TEBCs printed from different

anatomic sites showed significant differences ($p < 0.0001$) in morphometric parameters as determined by one-way ANOVA.

Scanning electron microscopy (SEM) was used to image trabecular interconnectivity and surface roughness of the TEBCs (Fig. 2A). SEM images revealed the presence of curved and interconnected trabeculae. Parallel ridges $\sim 25 \mu\text{m}$ apart were also observed, which were conjectured to result from the layer-by-layer fabrication of the wax templates. Optical profilometry was used to measure surface roughness compared to a dentin control (Fig. 2B). Dentin was chosen as a control due to its frequent use as a bone-like substrate for *in vitro* cell culture as well as its more reproducible surface properties compared to cortical bone.^[27–29] Flat molds were created using CAD software to allow for the printing of 2D PUR-nHA discs with layer thickness (and therefore surface roughness) comparable to that of the 3D TEBCs. The average surface roughness and water contact angle of the PUR-nHA films did not significantly differ from those of dentin, suggesting that the surface properties of the TEBCs are similar to those of bone (Fig. 2C). Fibronectin adsorption was significantly (two-fold) higher on dentin compared to the PUR-nHA film (Fig. 2C), which may be due to the absence of a collagen component in PUR-nHA.^[30] Bulk modulus, compressive strength, and yield strain of the TEBCs were measured by quasi-static mechanical testing in compression mode using an Instron® Testing System. Force-displacement curves were obtained from quasi-static compression testing of FH-, PT-, and VB-TEBCs and converted to stress-strain curves to determine bulk modulus, compressive strength, and yield strain (Fig. 2D). The bulk modulus of FH-TEBCs was within the range of values reported for trabecular bone from the proximal femur (21 – 965 MPa^[31]) and significantly higher than that of PT- and VB-TEBCs (Fig. 2E). The bulk moduli of PT- and VB-TEBCs were comparable to trabecular bone from the proximal tibia (5 – 552 MPa) and vertebral bodies (1.1 – 428 MPa).^[31] Since substrate modulus (E_s) could not be directly measured, it was calculated from the bulk modulus of each TEBC (K), polymer density ($\rho_s = 1.76 \text{ g cm}^{-3}$), and TEBC bulk density (ρ).^[32]

$$E_s = \frac{K}{(\rho/\rho_s)^2} \quad (1)$$

The calculated substrate moduli exhibited no significant differences between anatomic sites (Fig. 2E) and exceeded values measured for trabecular bone by nanoindentation ($365 \pm 223 \text{ MPa}$).^[31,33] This result suggests that the templating process does not adversely affect the microstructure of the PUR-nHA hybrid polymer, since the substrate modulus is independent of morphometric properties. Compressive strength (Fig. 2F) showed similar trends to that of the bulk modulus, with the femoral head exhibiting the highest compressive strength ($\sim 3 \text{ MPa}$), and all TEBCs falling within the range of reported compressive strength of trabecular bone (0.2 – 15 MPa). While yield strain followed similar trends (Fig. 2G), statistical significance was only observed between FH- and VB-TEBCs. Yield strains also fell within reported measurements of human bone and the specific anatomic sites (0.3–3%).^[34]

Cell culture experiments were performed to assess the ability of the TEBCs to support hMSC proliferation and osteogenic differentiation and determine the effects of

morphometric properties on cell behavior. hMSCs were suspended in growth medium (PromoCell), seeded on scaffolds in 30 μ L droplets (5×10^4 cells per TEBC), allowed to adhere for 3 hours prior to immersion in media, and cultured at 37°C for 24 h. Adherent cells were fixed and imaged by SEM, which showed cells attached and spread on all three anatomic sites. The MTS metabolic assay was conducted to compare cell proliferation and activity on the different TEBCs as well as 3D t-FDM scaffolds (Fig 3A), which were used as a control due to their controlled pore size and geometry (Fig 3H). Metabolic activity increased across all groups over a 5-day period; however, there were significant differences in activity between the groups. Metabolic activity was higher for anatomic sites with lower BV/TV, higher SMI, and higher Tb.Sp. (PT- and VB-TEBCs), with the PT-TEBCs showing significantly higher activity compared to FH-TEBCs and t-FDM scaffolds at all three time points. These findings suggest that the morphometric properties of trabecular bone regulate proliferation of osteoprogenitor cells.

Osteogenic differentiation of hMSCs was assessed by measuring expression of osteogenic genes using quantitative real-time PCR (polymerase chain reaction). Gene expression was assessed on D3, D5, and D8 after transfer to osteogenic differentiation medium (PromoCell) for the early marker of osteoblast differentiation alkaline phosphatase (*ALP*) and late-stage marker osteopontin (*OPN*) to investigate how morphometric properties regulate osteogenic differentiation. *ALP* expression significantly increased on D8 for hMSCs seeded on the more rod-like (more convex) PT- and VB-TEBCs, while those cultured on the more plate-like (less convex) FH-TEBCs and t-FDM scaffold showed decreasing *ALP* expression from D5 to D8 (Fig 3B). The late increase in *ALP* expression on the more rod-like PT- and VB-TEBCs compared to the more plate-like FH-TEBCs suggests that osteogenic differentiation is delayed for cells seeded on scaffolds with more convex surfaces. The late-stage marker *OPN* peaked at D8 for all constructs, indicating that the cells are approaching osteoblast maturation (Fig 3C).^[35] Furthermore, the largest fold change was observed for the t-FDM scaffolds. Concentrations of secreted osteocalcin (OCN), another late-stage osteoblast marker, were measured by ELISA (enzyme-linked immunosorbent assay) to further assess differences in osteogenic differentiation between anatomic sites (Fig 3D). A significant increase in OCN concentration was observed for VB-TEBCs on D8, while the other groups showed a decreasing trend by D8. This observation is in agreement with the *ALP* expression data suggesting that hMSCs cultured on the more convex VB-TEBCs exhibit delayed osteogenic differentiation compared to the less convex t-FDM scaffolds and FH-TEBCs.

Matrix mineralization was assessed to investigate how morphometric properties regulate osteoblast activity. Matrix mineralization after 7 and 10 days of culture in osteogenic medium was assessed by SEM (Fig 3E). D7 images indicate that cells are depositing matrix, but mineral nodules were observed only on t-FDM scaffolds and not the TEBCs. By D10, the SEM images exhibited extensive mineral nodules on t-FDM scaffolds and TEBCs. A representative high-magnification image of a nodule is shown in Fig. 3F, from which the average diameter was measured and compared to literature values. The average nodule size was found to be $8.7 \pm 6.4 \mu\text{m}$, which is within the reported range of nodule sizes ($\sim 10 \mu\text{m}$) at the early stages of mineralization.^[36] To provide further evidence of mineralization, TEBCs were stained on D7 and D10 by Alizarin Red, which reacts with calcium to form an Alizarin Red S-calcium complex. Cells were fixed in 10% formalin, stained, and extracted with 5%

SDS (sodium dodecyl sulfate) detergent to recover the stain. Absorbance of the extract was measured at 540 nm. As indicated by Alizarin Red absorbance, mineralization of t-FDM scaffolds on D7 was significantly higher ($p < 0.0001$) compared to the other groups, which showed minimal absorbance (Fig 3G). On D10, significant differences in mineralization were observed over time and between anatomic sites. From D7 to D10, significant increases in absorbance were observed for all groups except the VB-TEBCs, and the D10 absorbance was significantly different between each of the groups except the PT-TEBCs and VB-TEBCs ($p = 0.069$). These observations suggest that mineralization occurred between D7 and D10 for the TEBCs and that the degree of mineralization by D10 is dependent on scaffold architecture. Thus, the Alizarin Red staining data are consistent with the SEM images. Interestingly, mineralization on D10 (assessed by Alizarin Red absorbance) decreased significantly ($p < 0.01$) with increasing SMI (Fig 3H). SEM images of t-FDM scaffolds revealed the presence of completely enclosed concave pores (Fig. 3I). Consequently, the SMI of t-FDM scaffolds is close to zero as anticipated for structures with enclosed cavities.^[26,37] The observed increases in osteoblast differentiation and mineralization with decreasing surface convexity is consistent with previous studies reporting that the rate of new tissue formation on concave surfaces is significantly larger compared to convex or planar surfaces.^[38–40] While earlier studies have shown that surface curvature regulates osteogenesis *in vitro*^[41] and *in vivo*^[42], the effects of the anatomic site-specific morphometric properties of human trabecular bone on osteogenic differentiation have not been previously reported.

In summary, we report a new 3D inkjet printing process to fabricate anatomic site-specific TEBCs that recapitulate the morphometric and mechanical properties of human trabecular bone. Similar to bone, TEBCs comprised $> 50\%$ nanocrystalline hydroxyapatite. The morphometric parameters BV/TV, Tb.Sp., Tb.N. and SMI of TEBCs were comparable to the bone templates from which they were fabricated. Bulk modulus, compressive strength, and yield strain of the TEBCs were within the range of human bone, and the surface roughness was comparable to that of dentin. hMSCs cultured on TEBCs exhibited significantly different metabolic activities, osteogenic differentiation, and mineralization depending on the anatomic site. Proliferation increased with decreasing BV/TV and increasing Tb.Sp., while differentiation and mineralization increased with increasing BV/TV and decreasing Tb.Sp. The SMI, a measure of surface convexity, was identified as an important predictor of osteoblast mineralization. Taken together, these findings highlight the relative contribution of anatomic site-specific properties to osteoblast differentiation and mineralization, thereby supporting the need for anatomically relevant *in vitro* models. By recapitulating the morphometric properties of trabecular bone at specific anatomic sites in individual patients, bone-templated TEBCs will potentially provide a new platform technology for precision medicine approaches to treating diseases of the skeleton.

Experimental Section

Fabrication of TEBCs from Micro-computed Tomography Images: Human cadaver samples of the femoral head, proximal tibia, and vertebral body obtained from the Vanderbilt Anatomical Donation Program were scanned in a Scanco® μ CT50. Resulting image files were then inverted and converted to STL files using Scanco® Image Processing Language

(IPL). STL files were subsequently converted to Solidscape® 3Z Analysis software-compatible files and sent to a Solidscape® 3Z Studio Inkjet 3D printer to print a mold from the build material wax, a proprietary blend of sulfonamide derivatives. The resulting wax molds were submerged in leaching oil (Bioact VSO) to remove the support material, leaving behind the finished trabecular bone molds. The bone molds were then filled with a PUR-nHA hybrid composite material consisting of lysine diisocyanate (LDI)-grafted nHA and poly(caprolactone) triol (300 g mol^{-1} , Sigma) to yield a PUR-nHA network upon addition of FeAA catalyst, mixing, and curing at 50°C overnight. The wax mold was then leached from the PUR-nHA TEBCs by immersion in acetone. 2D PUR-nHA films with layer thicknesses representative of the 3D inkjet printer were cast into printed flat molds, cured, and leached in the same manner as previously described. TEBCs were soaked in ethanol overnight and placed under UV light for 30 minutes to sterilize prior to cell culture.

Contact Angle

Static water contact angle of 2D PUR-nHA films with characteristic 3D-printed surface roughness were compared to that of dentin using a contact angle goniometer. Contact angle was measured in 4 different locations on each film, and average values were calculated and compared between groups.

Mechanical Testing

TEBCs were tested in uniaxial compression mode using an Instron DynaMight 8800 Servohydraulic Test System (Norwood, MA) and operated in accordance with ASTM standard D695. The displacement rate was kept at a constant 1.3 mm/min , and compression was continued until failure.

Cell Culture

hMSCs (Extem Biosciences) were maintained in Mesenchymal Stem Cell Growth Medium 2 (PromoCell). Cells were detached at confluency by trypsin EDTA (Ethylenediaminetetraacetic acid, 0.25%) and resuspended at $1.67 \times 10^6 \text{ cells mL}^{-1}$ in same medium and seeded on TEBCs ($30 \mu\text{L scaffold}^{-1}$) pre-soaked in fibronectin solution ($4 \mu\text{g mL}^{-1}$) at 4°C for 24 h. After seeding, TEBCs were incubated for 3 h (5% CO_2 and 37°C) in 96-well plates before moving to new wells and adding $200 \mu\text{L}$ complete medium to facilitate cell attachment to the surface. Cell metabolism was measured by MTS Tetrazolium assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega). Absorbance values measured at optical density (OD) 490 nm were normalized to values measured for the t-FDM scaffolds on day 1 and reported as fold-change.

Osteogenic Gene Expression

hMSCs were seeded on TEBCs as described previously and cultured in Mesenchymal Stem Cell Growth Medium 2 (PromoCell) for 3 days, at which time the media was changed to osteogenic differentiation media (PromoCell). TEBCs were removed and immersed in 1 mL TRIzol (ThermoFisher) at D3, D5, and D8 after transfer to differentiation medium. RNA was isolated from TRIzol reagent by phase separation by addition of chloroform and subsequent RNA precipitation via addition of isopropanol. The resulting RNA pellet was

washed in 70% ethanol, air-dried, and re-suspended in RNase-free water. cDNA was synthesized from purified total RNA via iScript Reverse Transcription Supermix (Biorad). qPCR (Quant Studio 5, ThermoFisher) was used to assess expression of the osteogenic genes *ALP* and *OPN*. *ALP* (Hs01029144_m1) and *OPN* (Hs00959010_m1) primers were purchased from ThermoFisher. All reactions were run in triplicate and expression levels of *ALP* and *OPN* were normalized to 18s (ThermoFisher).

Mineralization

TEBCs seeded with hMSCs as described previously were cultured in osteogenic media for up to 10 days. They were then washed with phosphate-buffered saline (PBS, ThermoFisher), fixed in 10% formalin for 45 min, and stained with 20 mM Alizarin Red S for 5 min. After staining, TEBCs were washed ten times with DI water, and Alizarin Red S was extracted with 5% sodium dodecyl sulfate (SDS) for 1 hr. Absorbance of the extracted dye was read on a plate reader (OD, optical density = 540 nm), and dye extracted from cell-free TEBCs served as blank controls. For SEM, TEBCs were fixed in 5% glutaraldehyde, washed with DI water, fixed in 2% osmium tetroxide, and subsequently dried in increasing concentrations of ethanol and vacuum dried overnight. Samples were then cut to expose a variety of surfaces and mounted on a stub covered with carbon tape. Samples were gold sputter-coated (108 Auto Sputter Coater; TedPella, Redding, CA) and imaged using scanning electron microscopy (Carl Zeiss Inc, Thornwood, NY). Mineral nodule clusters were counted (> 50 clusters from > 20 SEM images) and characterized for average diameter using ImageJ software.

Statistical Analysis

The statistical analysis was performed by a one- or two-way analysis of variance (ANOVA). Graphs report the mean and standard deviation unless indicated otherwise. $P < 0.05$ is considered statistically significant with $n = 3$ for all experiments. All statistical analysis was performed using Prism 7 software.

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Figure 1.

Bone-templated constructs recapitulate the morphometric properties of human bone. (A) Micro-computed tomography (μ CT) images of human bone samples from three anatomical sites were inverted, segmented, converted to .stl files, and printed by a Solidscape® 3Z Studio inkjet printer to create wax template of human trabecular bone. Lysine methyl ester diisocyanate (LDI) was mixed with hydroxyapatite nanoparticles (nHA) and iron acetylacetonate (FeAA) catalyst to form an nHA-LDI dispersion that was subsequently mixed with a polyester triol and poured into the wax templates. After curing overnight, the wax templates were dissolved in acetone and the resulting constructs were washed in ethanol and subsequently DI water prior to characterization and cell culture. (B) μ CT images of the femoral head (FH), proximal tibia (PT), and vertebral body (VB) human bone templates (top row) and the TEBCs (bottom row). Scale bar represents 1 mm. (C) The morphometric parameters bone volume density (BV/TV), structure model index (SMI), trabecular separation (Tb. Sp.), and trabecular number (Tb. N.) were measured for host bone and TEBCs by μ CT. **** denotes statistical significance ($p < 0.0001$) measured by one-way ANOVA ($n = 8$).

Figure 2.

TEBCs recapitulate the surface and mechanical properties of human bone. (A) Representative SEM images of the TEBC trabecular structure at increasing magnification from left to right. (B) Optical profilometer topographical maps of dentin and TEBC surface (axes in μm) and comparison of surface roughness between dentin and TEBC. Arithmetic average roughness (R_a), quadratic average roughness (R_q) and max roughness height (R_t) were calculated from the optical profilometry data. (C) Water contact angle (measured by contact angle goniometer) and fibronectin adsorption on TEBCs compared to dentin control. (D) Example stress-strain curve of TEBCs under quasi-static compression testing. Elastic modulus, compressive strength, and yield strain were calculated as indicated. (E) Bulk (left y-axis) and substrate (right y-axis) moduli of TEBCs measured for each anatomic site. (F) Compressive strength and (G) yield strain of TEBCs templated from trabecular bone from different sites. ** ($p < 0.01$) and * ($p < 0.05$) denote statistical significance determined by one-way ANOVA ($n = 4$).

Figure 3.

Human bone marrow-derived stem cells (hMSCs) attach, proliferate, and differentiate toward an osteoblastic phenotype on TEBCs. (A) hMSCs were cultured on TEBCs for up to 5 days and metabolic activity determined by the MTS assay. Data are plotted as fold-change in absorbance at an optical density (OD) of 490 nm. (B-C) Expression of (B) *ALP* (early osteoblast marker) and (C) *OPN* (late osteogenic marker) measured by qRT-PCR for hMSCs cultured on TEBCs for up to 8 days. (D) Concentration of secreted osteocalcin (measured by ELISA) for hMSCs cultured on TEBCs for up to 8 days. (E) SEM images of hMSCs cultured in osteogenic medium for 7 days (top row) show adherent cells and mineral nodules (red arrows) on t-FDM constructs, but no mineral nodules were observed on the TEBCs. At day 10 (bottom row), all scaffolds showed evidence of mineralization. (F) Representative SEM image of mineral nodule on t-FDM scaffolds on day 10. (G) Absorbance of the Alizarin Red dye extracted from the mineralizing matrix on days 7 and 10. (H) Correlation of the degree of mineralization (assessed by Alizarin Red absorbance on day 10) with structure model index (SMI) of the TEBCs (Pearson's correlation coefficient; $r = -0.9916$, $p < 0.01$). (I) Low-magnification images of t-FDM scaffolds reveal the presence of enclosed cavities. All graphs show mean and SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$) determined by two-way ANOVA ($n = 3$).