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Fabrication of 3D Scaffolds with Precisely Controlled Substrate Modulus and Pore Size by Templated-Fused Deposition Modeling to Direct Osteogenic Differentiation

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The properties of the extracellular matrix, including elastic modulus, porosity, pore size, and curvature, are known to regulate cell fate in a number of physiological processes, including tissue repair and restoration as well as disease progression. Biomimetic 3D *in vitro* systems have been proposed for investigating interactions between cell populations and the

microenvironment (μ EN), identifying the molecular mechanisms of healing and/or disease progression, and screening drugs.^[1] Consequently, new biomaterials re-capitulating the bone μ EN are critical for understanding the spatio-temporal dynamics of bone remodeling as well as how bone cells integrate mechanical and chemical signals over multiple length scales. While Additive Manufacturing (AM) approaches such as Fused Deposition Modeling (FDM) enable precise control over topological properties^[2], the limited number of materials that can be processed by AM techniques precludes precise control over mechanical properties. Here, we fabricated 3D scaffolds with substrate moduli ranging from the basement membrane to the low end of cortical bone (10 – 900 MPa^[3, 4]) and pore sizes in the range that supports enhanced bone formation and vascularization ($>300\ \mu\text{m}$ ^[5, 6]) using a new templated-Fused Deposition Modeling (t-FDM) process. Previous studies have reported that osteogenic differentiation of mesenchymal stem cells (MSCs) increases with substrate modulus up to about 100 kPa.^[7] However, it has been suggested that above this range cells are in an isometric state of contraction and cannot respond to further increases in rigidity.^[8, 9] Surprisingly, we found that osteogenic differentiation and mineralization of MSCs increased with increasing substrate modulus in the MPa range and decreasing pore size.

A limited number of studies have investigated the effects of the elastic modulus on osteogenic differentiation on rigid substrates approximating trabecular bone (100 – 400 MPa^[3]). Expression of the osteogenic transcription factor *Runx2* and the osteoblast marker alkaline phosphatase (*ALP*) increased with 2D substrate rigidity when MC3T3-E1 pre-osteoblasts were cultured on PEG-diacrylate hydrogels (600 kPa) or tissue culture polystyrene (2000 MPa).^[10] RhoA activity increased on stiffer substrates, thereby promoting increased cellular contractility through ROCK. A later study reported that for 2D acrylate films with moduli ranging from 5–850 MPa, the composition of the polymer had a more significant effect on differentiation than the substrate modulus.^[3] Curvature, pore size, and pore shape have also been reported to affect the rate of new bone formation. A recent study has reported that new bone formation is enhanced on concave versus convex or flat surfaces.^[11] Cells respond to radii of curvature larger than themselves, and the magnitude of the contractile forces and the rate of new bone formation have been suggested to increase with the degree of curvature of the surface.^[12, 13] This increasing osteogenic potential with increasing curvature has been attributed in part to mechanical forces^[12], since smaller pores with a higher degree of curvature may result in increased stress concentration.^[13] Thus, the effects of substrate modulus and pore size on osteogenesis may be inter-related. While previous studies point to substrate modulus, pore size, and pore shape as determinants of osteogenic differentiation, the relative contributions of these mechanical and topological properties to osteogenic differentiation have not been fully elucidated. A more fundamental understanding of how the properties of the matrix direct osteogenesis would guide the rational design of cell-responsive scaffolds that recapitulate the bone μ EN for restoration and repair of bone damaged by trauma or disease.

To fabricate t-FDM scaffolds with well-defined topological properties approximating those of rod-like trabecular bone, polylactic acid (PLA) templates were designed using Autodesk software and fabricated by Fused Deposition Modeling (FDM) with a MakerBot[®] Replicator 2 3D printer (Figure 1A). PLA templates were printed with 100% inter-connected nominal

300 μm (Figure 1B) or 500 μm (Figure 1C) fibers. The smaller pore size was selected to be representative of that optimal for osteogenesis (300 – 400 μm ^[14]), while the larger pore size was selected to be representative of diseased bone (trabecular separation ranging from 430 – 930 μm ^[15]). Measured PLA fiber sizes were 423 ± 34 and 557 ± 44 μm for nominal 300 and 500 μm templates, respectively (Figure 1D). The reactive polyurethane (PUR), which comprised hexamethylene diisocyanate trimer (HDI_t), a polyester triol (300 (Rigid, R) or 3000 (Compliant, C) g mol^{-1}), and iron acetylacetonate (FeAA) catalyst, was mixed and poured into the PLA template. The catalyst concentration was adjusted to maintain the reactive PUR as a fluid for a sufficient period of time required to fill the inter-fiber space. After curing overnight, PLA fibers were leached from the scaffolds by immersion in dichloromethane for 18h to form inter-connected pores in the PUR scaffolds, while the inter-fiber space was filled with PUR (Figure 1E). To determine the effects of scaffold processing on PUR composition and surface chemistry, rigid and compliant 2D PUR films were also synthesized by casting in well plates. FTIR analysis showed absorbance of the carbonyl stretch peak (1680 – 1710 cm^{-1}) associated with ester, urethane, and isocyanurate bonds, as well as absorbance of the isocyanurate ring structure in HDI_t at 1410 – 1450 cm^{-1} .^[16] Water contact angles and fibronectin adsorption (measured by fluorescent labeling^[17]) showed no significant differences between rigid and compliant groups (Figure 1F), indicating similar surface chemistry for protein adsorption and consequent cell adhesion.

The ratio of fiber diameter:inter-fiber spacing was maintained at 1:1 in order to provide relatively constant porosity ($46 \pm 4\%$ to $52 \pm 2\%$) and $\sim 100\%$ inter-connected pores across scaffold groups. Substrate modulus was controlled by synthesizing PUR scaffolds from polyester triols of different molecular weight (300 (R) or 3000 (C) g mol^{-1}) and was measured using two independent techniques: nanoindentation and compression. Substrate nanoindentation modulus (E_s) was determined using the method of Oliver and Pharr^[18]. While the values of E_s matched for compliant 2D films and 3D scaffolds (10 MPa), E_s was $\sim 30\%$ higher for the rigid films compared to the scaffolds (1700 MPa versus 1000 MPa, Figure 1G). The bulk substrate modulus (K_s) of the 2D films and the bulk scaffold modulus (K^*) were also measured under compression by MTS.^[19] However, unlike nanoindentation, K_s cannot be measured directly for 3D scaffolds, and was therefore calculated ($K_{s,\text{pred}}$) from measured values of the scaffold bulk modulus (K^*), polymer density ($\rho = 1.27 \text{ g cm}^{-3}$), and scaffold porosity ε ^[20]:

$$K_{s,\text{pred}} = K^* [\rho / (1 - \rho\varepsilon)]^2 \quad (1)$$

No significant differences were observed between measured (K_s) and calculated ($K_{s,\text{pred}}$) values of the bulk substrate modulus (Figure 1H), which suggests that the substrate modulus for each polymer (rigid or compliant) is similar in 2D and 3D. However, K_s was significantly higher for rigid (250 – 300 MPa) compared to compliant (~ 5 MPa) scaffolds. The bulk substrate modulus was generally lower than the nanoindentation substrate modulus, which is attributed to differences in the applied modes of deformation.

Cell culture experiments were performed to investigate the effects of elastic modulus and pore size on adhesion, proliferation, and differentiation of bone marrow-derived

mesenchymal stem cells (BMSCs). Rat BMSCs were suspended in complete cell culture medium, seeded onto scaffolds (10^5 cells cm^{-3} scaffold), and cultured at 37°C for 24 h. Adherent cells imaged by scanning electron microscopy (SEM) were well spread on all four scaffolds (Figure 2A), which is consistent with the notion that cells cannot displace substrates with elastic moduli exceeding 100 kPa.^[8, 9] To investigate the effects of modulus and pore size on viability, live/dead staining, total protein assays, and metabolic assays were performed. Minimal (<5%) cell death was detected 48 h after cell seeding in all four groups (Figure 2B), indicating that the scaffolds are non-cytotoxic. Cell proliferation was assessed by total protein and metabolic assays. While total protein increased from day 1 to day 7 for all treatment groups (Figure 2C), differences between groups were not significant, which suggests that modulus and pore size have minimal effects on proliferation. In contrast, the metabolism level of BMSCs cultured in rigid scaffolds was significantly higher than that observed for compliant scaffolds at each time point, while pore size effects were not significant (Figure 2D). These observations suggest that cells are more metabolically active on rigid substrates, which was conjectured to promote increased cell migration. Two assays were performed to assess the effects of substrate modulus on migration of BMSCs. Live cell tracking^[21] on 2D rigid and compliant PUR films showed that BMSCs migrated faster on rigid films (Figure 2E). Due to technical challenges associated with tracking cells in 3D, trans-well invasion experiments^[22] were performed using 3D scaffolds, which showed that cells migrated faster on rigid scaffolds, although differences for 500 μm scaffolds were not significant (Figure 2F–G). These findings suggest that metabolism and migration of BMSCs at early time points after seeding (< 7 days) are primarily controlled by the substrate modulus.

Rat BMSCs were cultured in osteogenic medium (complete cell culture medium supplemented with 10 nM dexamethasone, $50 \mu\text{g ml}^{-1}$ ascorbic acid, and 0.1 mM β -glycerophosphate) for up to 21 days and total RNA extracted to quantify expression of osteogenic genes at D5, 10 and 15. These time points, which differ from those commonly used to assess osteogenic differentiation (D4, 7, 14, and 21^[23]), were selected based on preliminary time course experiments. Expression of the transcription factor *Runx2*^[24] was assessed by quantitative real-time PCR (qPCR) to investigate the effects of scaffold properties on osteogenic differentiation of BMSCs. While the effects of pore size on expression of *Runx2* were not significant, *Runx2* was significantly up-regulated on rigid scaffolds at 5 and 15 days (Figure 3A). Expression of *Collagen-1* (*COL1A1*) and *Fibronectin* (*FNI*) has been reported to be necessary for osteogenic differentiation^[25] and was also evaluated in this study. Significant increases in gene expression were observed on D5, with increased *COL1A1* expression observed for 500-R and 300-R (Figure 3B) and increased *FNI* expression observed for 300-R (Figure 3C). These observations are consistent with a recent study reporting that *COL1A1* and *FNI* are early markers of osteogenic differentiation^[26], and therefore point to an early cellular response to substrate rigidity that drives increased production of extracellular matrix proteins to support osteogenic differentiation of BMSCs.^[27] Expression of *Osteopontin* (*OPN*), a prominent component of the mineralized extracellular matrix in bone and a marker of osteogenic differentiation^[28], was also evaluated in this study. *OPN* expression was significantly higher on rigid scaffolds at D10 and D15 (Figure 3D). Expression was significantly higher on 300-R compared to

500-R scaffolds on D10, but the effects of pore size on *OPN* expression were insignificant on D15.

Since the ability to mineralize the extracellular matrix is an important late-stage marker of osteogenic differentiation, mineralization was assessed by the deposition of aggregated mineral nodules.^[23] Cells were fixed, stained for nodular aggregates at D21 by Alizarin Red S^[29], the adsorbed dye extracted from the scaffolds with 5% SDS solution^[30], and the absorbance of the extract measured at 550 nm. Consistent with the gene expression data, Alizarin Red S absorbance increased with scaffold rigidity (Figure 3E), indicating up-regulated mineralization on the rigid scaffolds. Interestingly, mineralization also significantly increased with decreasing pore size. Another group of scaffolds were fixed at D21, sectioned (5 μm), and stained with Alizarin Red S. A greater number of mineralized nodules were observed on the rigid compared to compliant scaffolds (Figure 3F). Furthermore, SEM images of mineralized extracellular matrix at D21 (Figure 3G) showed larger and more extensive formation of mineral nodules on 300-R compared to the other scaffolds. Taken together, the gene expression and mineralization experiments show that osteogenic differentiation was highest on the rigid scaffolds with bone-like substrate modulus and 423 μm pores, which challenges the notion that cells adhered to substrates with moduli exceeding 100 kPa are in an isometric state of contraction. Considering that cells cannot displace substrates with moduli greater than about 10 kPa^[9], an alternative mechanotransduction mechanism, such as coupling of an integrin and soluble factor receptor^[31], is conjectured to promote the observed increased osteogenesis on rigid bone-like substrates.

There are a limited number of studies investigating the effects of scaffold substrate modulus and pore size on osteogenic differentiation using AM approaches that enable precise control over mechanical and topological properties.^[2] Our observation that mineralization increased with decreasing pore size is consistent with a recent study reporting that scaffolds fabricated by selected laser melting (another AM technique) with 500 μm pores enhanced cellular response compared to 1000 μm .^[32] Interestingly, the majority of *in vivo* studies have reported enhanced new bone formation in scaffolds with larger pore sizes.^[2, 6, 33] Thus, while effects such as stress concentration^[13] may increase osteogenic potential *in vitro* in scaffolds with smaller pores, the relative contributions of phenomena such as reduced transport^[34] and initiation of new bone formation through a chondrogenic pathway^[33, 35] may be more important *in vivo*. The topology of the scaffold may also affect osteogenesis and new bone formation. Trabecular bone exhibits both rod- and plate-like structures, and thus the degree of curvature varies spatially within the bone.^[36] Furthermore, the relative portions of rods and plates, which is quantified by the Structure Model Index (SMI) describing the trabecular architecture^[36], varies from one anatomic site to another. Thus, further *in vivo* studies are needed to identify how new bone formation is controlled by substrate pore size, pore shape, and surface curvature, which can be precisely controlled by templated AM techniques.

In this study, we designed a new t-FDM process to fabricate 3D scaffolds with tunable substrate modulus over the range of 10 – 900 MPa, which spans the complete range of substrate moduli for trabecular bone (93 – 365 MPa^[3]) and the lower end of the range for

cortical bone (871 – 11,500 MPa^[3]). Osteogenic differentiation and mineralization increased as the substrate modulus increased from 10 to 900 MPa, which has not been previously reported in 3D. Mineralization also increased as pore size decreased from 557 to 423 μm . While the t-FDM scaffolds described herein exhibited precisely controlled topological properties with ~100% interconnectivity, the patterned, rod-like pores of the t-FDM scaffolds differ from the irregular pores of trabecular bone. Future studies aim to design templated AM scaffolds with a mixture of rods and plates, which more accurately recapitulates the topological properties of trabecular bone.

Experimental section

Fabrication and characterization of PUR scaffolds from PLA templates

PLA templates were designed using Autodesk and fabricated by FDM (MakerBot Replicator[®] 2 3D printer). Polyester triols (300 g mol⁻¹ or 3000 g mol⁻¹) were synthesized from a glycerol starter and a backbone comprising ϵ -caprolactone, glycolide, and D,L-lactide.^[37] PUR scaffolds were synthesized by reactive liquid molding of HDIt, the polyester triol, and FeAA catalyst (5% FeAA in dipropylene glycol), which were poured into the PLA templates (14 mm diameter) after mixing and cured at 60°C overnight. PLA templates were then removed by extraction with dichloromethane overnight. Pore size was controlled by adjusting the diameter of the PLA fibers, which was measured by SEM for 50 PLA fibers in each scaffold.

Cell culture

Primary rat bone marrow mesenchymal stem cells (BMSCs) were generated from pooled bone marrow from femurs of four Sprague-Dawley rats. All surgical and care procedures were carried out under aseptic conditions per an approved IACUC protocol. BMSCs were maintained in DMEM with 10% FBS, 1% P/S, and 0.1% Amphotericin B (Sigma). Cells were detached at sub-confluency by trypsin EDTA (0.25%) and re-suspended at 10⁶ cells/mL in complete medium and seeded on scaffolds (60 μl /scaffold) pre-soaked in fibronectin solution (4 $\mu\text{g/mL}$) at 37°C for 24 h. After seeding, scaffolds were incubated for 4h (5% CO₂ and 37°C) in 12-well plates before adding 2mL complete medium to facilitate cell attachment to the surface. A Live/Dead[®] Viability/Cytotoxicity Kit (Invitrogen) was used to measure cell viability 48h after seeding. Cell proliferation and metabolism were measured by total protein (BCA Protein Assay Reagent, Thermo) and MTS assay (CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay, Promega) respectively.

Cell migration assay

BMSCs plated on 2D PUR films were cultured in a live cell chamber (LiveCell[™]) at 5% CO₂ and 37°C and monitored by light microscopy (Olympus CKX41). Photos of the same field were taken every 30 min and the photo series analyzed by Image J to track single cell movement. The 3D BMSC invasion assay was conducted using a cell transwell plate (Corning). Cells were cultured on 3D scaffolds (3 mm height $\times$ 6 mm diameter) as described above and placed in the transwell inserts. Cells were attracted to the bottom by serum gradient. Complete medium was added to the outer well while reduced serum was used in

the transwell to establish a serum gradient. After 72 h, the inserts were removed and the number of cells attached to the bottom plate counted by optical microscopy.

Osteogenesis assays

BMSCs were cultured in complete medium after seeding for 4 days and changed to osteoinductive medium for osteoblast differentiation (10 nM dexamethasone, 50 $\mu\text{g ml}^{-1}$ ascorbic acid and 0.1 mM β -glycerophosphate). Cells were detached by trypsin EDTA (0.25%) at D5, D10, and D15, and total RNA was isolated from the harvested cell pellets by RNeasy mini Kit (Qiagen). cDNA synthesis was carried out from purified total RNA using iScriptTM Reverse Transcription Supermix (Biorad). Quantitative real-time PCR (qPCR) for osteogenic genes was performed to assess osteoblast differentiation. The following primers were used: *Runx2*: TCCAGACCAGCAGCACTCC (forward 5' to 3'), GTTATGAAAAACCAAGTAGCCAGGT (reverse 5' to 3'); *COL1A1*: TGCTGATGGACAACCTGG (forward 5' to 3'), ACTGTTGCCTTTGGGACC (reverse 5' to 3'); *OPN*: AGTGGTTTGCCTTTGCCTGTT (forward 5' to 3'), TCAGCCAAGTGGCTACAGCAT (reverse 5' to 3'); *FN1*: GCACAGGGGAAGAAAAGGAG (reverse 5' to 3'), TTGAGTGGATGGGAGGAGAG (reverse 5' to 3'). The PCR reactions were amplified for 40 cycles at 95°C for 3 min, 95°C for 10 s, and 52°C for 30 s. All reactions were run in triplicate for each sample and the qPCR results for each gene were normalized to GAPDH.

Mineralization

Scaffolds were maintained in osteogenic media for up to 21 days, washed with DPBS (Gibco), and fixed in formalin (10%). Alizarin Red S staining was applied after fixation. In one cohort, dyes were dissolved in 5% SDS immediately following staining, and the absorbances of the resulting solutions were read on a plate reader (OD=550nm). In another cohort, scaffolds were fixed in 10% formalin for 15 minutes, washed in PBS, and dehydrated through increasing alcohol concentrations (50–100%). Fixed scaffolds were embedded in poly(methyl methacrylate) (PMMA), cut into 5 μm sections, and stained with Alizarin Red S for histological evaluation of mineralization. In a third cohort, scaffolds were cut to expose the center surfaces, fixed as described above, air-dried, and mounted to a specimen stub using carbon tape. Samples were sputter-coated with gold (108 Auto Sputter Coater; TedPella, Redding CA) and viewed via scanning electron microscopy (Hitachi, Finchampstead, UK).

Statistical Analysis

The statistical significance between experimental groups was determined by Student's *t test* or by a two-factor ANOVA. Graphs show mean and S.D., and $p < 0.05$ is considered statistically significant.

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References

- Schuessler TK, Chan XY, Chen HJ, Ji K, Park KM, Roshan-Ghias A, Sethi P, Thakur A, Tian X, Villasante A, Zervantonakis IK, Moore NM, Nagahara LA, Kuhn NZ. *Cancer Res.* 2014; 74:5359. [PubMed: 25095784]
- Zadpoor AA. *Biomaterials Science.* 2015; 3:231. [PubMed: 26218114]
- Smith KE, Hyzy SL, Sunwoo M, Gall KA, Schwartz Z, Boyan BD. *Biomaterials.* 2010; 31:6131. [PubMed: 20510445]
- Parekh A, Ruppender NS, Branch KM, Sewell-Loftin MK, Lin J, Boyer PD, Candiello JE, Merryman WD, Guelcher SA, Weaver AM. *Biophys J.* 2011; 100:573. [PubMed: 21281571]
- Rossi F, Santoro M, Perale G. *J Tissue Eng Regen Med.* 2013; doi: 10.1002/term.1827Cyster LA, Grant DM, Howdle SM, Rose FR, Irvine DJ, Freeman D, Scotchford CA, Shakesheff KM. *Biomaterials.* 2005; 26:697. [PubMed: 15350773] Penk A, Forster Y, Scheidt HA, Nimptsch A, Hacker MC, Schulz-Siegmund M, Ahnert P, Schiller J, Rammelt S, Huster D. Magnetic resonance in medicine: official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine. 2013; 70:925.
- Karageorgiou V, Kaplan D. *Biomaterials.* 2005; 26:5474. [PubMed: 15860204]
- Engler AJ, Sen S, Sweeney HL, Discher DE. *Cell.* 2006; 126:677. [PubMed: 16923388] Guvendiren M, Burdick JA. *Nature communications.* 2012; 3:792.Rowlands AS, George PA, Cooper-White JJ. *Am J Physiol Cell Physiol.* 2008; 295:C1037. [PubMed: 18753317]
- Discher DE, Janmey P, Wang YL. *Science.* 2005; 310:1139. [PubMed: 16293750]
- Moore SW, Roca-Cusachs P, Sheetz MP. *Dev Cell.* 2010; 19:194. [PubMed: 20708583]
- Khatriwala CB, Kim PD, Peyton SR, Putnam AJ. *J Bone Miner Res.* 2009; 24:886. [PubMed: 19113908] Khatriwala CB, Peyton SR, Metzke M, Putnam AJ. *J Cellular Physiology.* 2007; 211:661.
- Bidan CM, Kommareddy KP, Rumpler M, Kollmannsberger P, Fratzl P, Dunlop JW. *Advanced healthcare materials.* 2013; 2:186. [PubMed: 23184876]
- Rumpler M, Woesz A, Dunlop JW, van Dongen JT, Fratzl P. *J R Soc Interface.* 2008; 5:1173. [PubMed: 18348957]
- Sanz-Herrera JA, Moreo P, Garcia-Aznar JM, Doblare M. *Biomaterials.* 2009; 30:6674. [PubMed: 19781764]
- Lee KW, Wang S, Lu L, Jabbari E, Currier BL, Yaszemski MJ. *Tissue Eng.* 2006; 12:2801. [PubMed: 17518649]
- Marinozzi F, Marinozzi A, Bini F, Zuppante F, Pecci R, Bedini R. *Annali dell'Istituto superiore di sanita.* 2012; 48:19.
- Flores M, Fernandez-Francos X, Morancho JM, Serra A, Ramis X. *J Appl Polym Sci.* 2012; 125:2779.Caille D, Pascault JP, Tighzert L. *Polym Bull.* 1990; 24:23.
- Ruppender NS, Merkel AR, Martin TJ, Mundy GR, Sterling JA, Guelcher SA. *PLoS ONE.* 2010; 5:e15451. [PubMed: 21085597]
- Oliver WC, Pharr GM. *Journal of Materials Research.* 1992; 7:1564.
- Dumas JE, Zienkiewicz K, Tanner SA, Prieto EM, Bhattacharyya S, Guelcher S. *Tissue Eng Part A.* 2010; 16:2505. [PubMed: 20218874]
- Gibson, LJ.; Ashby, MF. *Cellular solids: Structure and properties.* Cambridge University Press; 1997.
- Meijering E, Dzyubachyk O, Smal I. *Methods Enzymol.* 2012; 504:183. [PubMed: 22264535]
- Soroceanu L, Manning TJ Jr, Sontheimer H. *The Journal of neuroscience: the official journal of the Society for Neuroscience.* 1999; 19:5942. [PubMed: 10407033]
- Barati B, Walters JD, Shariati SRP, Moeinzadeh S, Jabbari E. *Langmuir.* 2015; 31:5130. [PubMed: 25879768]

24. Gersbach C, Le Doux J, Guldberg R, García A. *Gene Ther.* 2006; 13:873. [PubMed: 16496016]
Bruderer M, Richards RG, Alini M, Stoddart MJ. *Eur Cell Mater.* 2014; 28:269. [PubMed: 25340806]
Lee JS, Lee JM, Im GI. *Biomaterials.* 2011; 32:760. [PubMed: 20947160]
25. Wang X, Schröder H, Grebenjuk V, Diehl-Seifert B, Mailänder V, Steffen R, Schloßmacher U, Müller W. *Mar Drugs.* 2014; 12:1131. [PubMed: 24566262]
Hamidouche Z, Fromigüé O, Ringe J, Häupl T, Vaudin P, Pagès J, Srouji S, Livne E, Marie P. *Proc Natl Acad Sci U S A.* 2009; 106:18587. [PubMed: 19843692]
26. Chen D, Shen H, He Y, Chen Y, Wang Q, Lu J, Jiang Y. *Molecular Medicine Reports.* 2015; 11:1111. [PubMed: 25373389]
27. Park JS, Chu JS, Tsou AD, Diop R, Tang Z, Wang A, Li S. *Biomaterials.* 2011; 32:3921. [PubMed: 21397942]
Karamichos D, Skinner J, Brown R, Mudera V. *J Tissue Eng Regen Med.* 2008; 2:97. [PubMed: 18338818]
28. Chen Q, Shou P, Zhang L, Xu C, Zheng C, Han Y, Li W, Huang Y, Zhang X, Shao C, Roberts AI, Rabson AB, Ren G, Zhang Y, Wang Y, Denhardt DT, Shi Y. *Stem Cells.* 2014; 32:327. [PubMed: 24123709]
Lee J, Abdeen AA, Kilian KA. *Sci Rep.* 2014; 4:5188. [PubMed: 24898422]
Sodek J, Ganss B, McKee MD. *Osteopontin.* 2000; 11:279.
29. Talukdar Y, Rashkow J, Lalwani G, Kanakia S, Sitharaman B. *Biomaterials.* 2014; 35:4863. [PubMed: 24674462]
30. Alford A, Terkhorn S, Reddy A, Hankenson K. *Bone.* 2010; 46:464. [PubMed: 19744582]
Su N, Sun Q, Li C, Lu X, Qi H, Chen S, Yang J, Du X, Zhao L, He Q, Jin M, Shen Y, Chen D, Chen L. *Human molecular genetics.* 2010; 19:1199. [PubMed: 20053668]
31. Galliher AJ, Schiemann WP. *Breast Cancer Res.* 2006; 8:R42. [PubMed: 16859511]
32. Van Bael S, Chai YC, Truscetto S, Moesen M, Kerckhofs G, Van Oosterwyck H, Kruth JP, Schrooten J. *Acta Biomater.* 2012; 8:2824. [PubMed: 22487930]
33. Jin QM, Takita H, Kohgo T, Atsumi K, Itoh H, Kuboki Y. *J Biomed Mater Res.* 2000; 52:841.
34. Takahashi Y, Tabata Y. *J Biomater Sci Polym Ed.* 2004; 15:41. [PubMed: 15027842]
35. Kuboki Y, Jin Q, Takita H. *J Bone Joint Surg Am.* 2001; 83-A(Suppl 1):S105. [PubMed: 11314788]
36. Hildebrand T, Rueggsegger P. *Computer methods in biomechanics and biomedical engineering.* 1997; 1:15. [PubMed: 11264794]
37. Guelcher SA, Patel V, Gallagher KM, Connolly S, Didier JE, Doctor JS, Hollinger JO. *Tissue Engineering.* 2006; 12:1247. [PubMed: 16771638]

Figure 1. Fabrication and characterization of 3D scaffolds with tunable elastic moduli and pore size by template Fused Deposition Modeling (t-FDM)

(A) Hexamethylene diisocyanate trimer (HDIt) was mixed with a polyester triol and iron acetylacetonate (FeAA) catalyst and poured into a poly(lactic acid) (PLA) template fabricated by a MakerBot® Replicator 2 3D printer. After curing overnight, the PLA template was dissolved in dichloromethane for 18 h. After washing with DI water, cells were cultured on the scaffolds. (B–C) SEM images of the nominal (B) 300 and (C) 500 μm PLA templates. (D) Size of PLA fibers in the scaffolds measured by SEM. (E) SEM images of 500C, 300C, 500R, and 300R scaffolds. (F) Water contact angle and Fn adsorption (arbitrary fluorescence units) measured for 2D compliant (C) and rigid (R) films. (G) Nanoindentation substrate modulus (E_s) of PUR films (2D) and 300C and 300R scaffolds (3D). (H) Comparison of substrate bulk modulus measured for films (K_s 2D) and predicted ($K_{s,pred}$ 300 and $K_{s,pred}$ 500) from the measured bulk modulus of 3D scaffolds (K^*) and cellular solids theory. * $p < 0.05$, ** $p < 0.01$.

assessed by an invasion assay, increased with increasing substrate modulus and decreasing pore size. (G) Images (10×) of cells stained with crystal violet that migrated through the scaffolds to the well plate in the invasion assay. * $p < 0.05$.

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Figure 3. Osteogenic differentiation and mineralization increase with increasing substrate modulus and decreasing pore size of t-FDM scaffolds

(A–D) Gene expression measured by qPCR. (A) *Runx2* (osteogenic transcription factor) expression is highest on 500R and 300R scaffolds on D5 and D15. (B) *COL1A1* expression is significantly higher on rigid scaffolds on D5. (C) *FN1* expression is highest on 300R scaffolds on D5. (D) *OPN* (late marker of osteogenic differentiation) expression is highest on rigid scaffolds on D10 and D15, and highest on 300R scaffolds on D10. (E) Alizarin Red S absorbance measured at 550 nm increases significantly with increasing substrate modulus and decreasing pore size. (F) High-magnification (40×) images of histological sections stained with Alizarin Red S show more extensive mineralized nodules (black arrows) on rigid versus compliant scaffolds. (G) SEM images show larger and more extensive mineralized nodules on 300R scaffolds (yellow arrow), as well as cells embedded in extracellular matrix (white arrows). * $p < 0.05$.