ADVANCED HEALTHCARE MATERIALS

Supporting Information

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Hierarchical Fabrication of Engineered Vascularized Bone Biphasic Constructs via Dual 3D Bioprinting: Integrating Regional Bioactive Factors into Architectural Design

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Experimental Section

Design and Fabrication of Hierarchical Construct: In order to mimic native bone, the "honeycombed pore shaped" scaffolds were composed of stacked units with a 200 μm line distance and a 200 μm layer height to form a porous cylinder, which is similar to the cylindrical structures of the osteon or haversian system. In addition, a series of interconnected horizontal and vertical vascular channels were designed to provide a biomimetic fluid environment for *in vitro* study and invasion spaces of native blood vessels for *in vivo* implantation. A 2 mm marrow cavity-like vascular channel is located in the center of construct and eight 500 μm diameter vascular channels pass through the whole bone regions of the scaffold allowing the culture medium unobstructed infusion.

This 3D model was fabricated into a biphasic scaffold through a dual bioprinting manufacturing technology employed in a two-step manner. A table-top FDM 3D bioprinter and a SLA based 3D bioprinter were developed in our lab based on the existing rapid prototyping platforms for use as a proof-of-concept 3D bioprinting system for advanced manufacturing of hierarchical constructs. The hard bone regions in the constructs were fabricated first using PLA on the FDM bioprinter. Next, the SLA bioprinter (laser beam is about 190 μm, wavelength is about 355 nm and the intensity output of emitted UV is ~20 μJ

at 15 kHz) was used to print the elastic vascular structure by infilling the interconnected channels and pores with cell-laden GelMA hydrogel filling the remaining spaces in the construct. In order to obtain a cylindrical channel in the hydrogels, a needle-based subtractive technique was performed to extract a lumen structure in the cell-laden hydrogel using a stainless steel microneedle. GelMA was synthesized as photocurable bioink for SLA bioprinting.[1] Briefly, methacrylic anhydride (MA) (1% (v/v), Sigma–Aldrich) was added dropwise to the gelatin (Sigma–Aldrich, 10% in PBS (w/w)) solution while stirring, and then the mixture was reacted for 3 h at 50 °C. The GelMA solution was dialyzed against deionized water for 7 d at 50 °C. The dialyzed GelMA solutions were lyophilized, and stored at room temperature. Before use, a GelMA polymer solution was prepared by dissolving the freeze-dried GelMA and the photoinitiator (Irgacure 2959) (0.5 w/v%) in PBS (0.01 M). Finally, representative CAD models and Slic3r configurations of the hierarchical constructs was used to analyze and calculate all structural parameters, including the wall thickness, pore size, porosity, and channel size.

Regional Fabrication of Bioactive Factors: During the 3D scaffold printing process, the osteogenic and angiogenic peptides were immobilized onto corresponding regions, respectively. The peptide sequence, KIPKASSVPTELSAISTLYLNH_2, represents a specific domain of BMP2. A cysteine amino acid at the N-terminus of this sequence was designed and introduced to allow its further reaction with the other active group. Through the same method, another peptide with a thiol group at the N terminus was also designed and prepared to mimic VEGF protein with the peptide sequence, KLTWQELYQLKYKGINH_2. Peptides were obtained with more than 95% purity according to the HPLC profile provided by the manufacturer (GenScript). BMP2 peptide was bonded on the PLA scaffold's surface by mussel-inspired chemistry. In brief, PLA scaffolds were immersed in a dopamine (DA) solution (2 mg/mL in 10 mM Tris-HCl, pH 8) and placed on a shaker for 12 h in the presence

of oxygen at room temperature. After 12 h, they were rinsed with distilled water five times, and then the polydopamine (pDA)-coated scaffolds were immersed in 20 ng/mL BMP2 peptide solution. Finally, the scaffolds were washed with distilled water three times to remove the unattached peptides. Correspondingly, VEGF peptide (50 ng/mL) was conjugated into the hydrogel via click chemistry during SLA bioprinting. ATR-FTIR spectroscopy measurements were performed with a Perkin Elmer Spectrum BX system, to detect changes of all components' structure in engineered scaffold construction.

Mechanical and Morphological Characterization of Constructs: The mechanical properties of all scaffolds were tested using a MTS criterion universal testing system equipped with a 100 N and 50 kN load cell (MTS Corporation, US), according to International Organization for Standardization (ISO) and American Society for Testing and Materials (ASTM). The scaffolds were compressed at a strain rate of 2 mm/min to a maximum strain of 20%. The slope of the linear elastic region of the stress-strain curve was calculated to obtain the compressive modulus. The morphology and surface topography of constructs for both the PLA scaffold and the GelMA gel segments were studied using a Zeiss SigmaVP scanning electron microscope (SEM). All scaffolds were coated with a 10 nm thick gold layer and imaged using a 5 kV electron beam.

hMSCs and HUVECs Culture Protocol: hMSCs (Texas A&M Health Science Center, Institute for Regenerative Medicine) were cultured in mesenchymal stem cell growth medium (MSCGM) consisting of alpha minimum essential medium, 20% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. HUVECs (Life Technologies) were cultured in endothelial growth medium (EGM) consisting of Medium 200 and low serum growth supplement (LSGS). For osteogenic differentiation studies, hMSCs were cultured in osteoinductive medium (OM, MSCGM supplemented with 10 nM dexamethasone, 50 μg/mL

L-ascorbate acid and 10 mM β-glycerophosphate (Sigma)). All experiments were performed with hMSCs and HUVECs of six cell passages or less.

Cell Adhesion, Encapsulation and Proliferation: To study the effect of pDA and BMP2 peptides on hMSC attachment, the cells (2×10^5 cell/mL) were seeded on various scaffolds for 4 h. The samples were assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT solution (0.5 mg/mL) was added in the plate and then incubated for 4 h. After the medium was removed, isopropanol/HCl solution (1 M) was added to dissolve the formazan crystals. The optical density (OD) was measured at 490 nm by photometric plate reader (Thermo Scientific). The cell proliferation on these scaffolds was conducted for 1, 3 and 5 days. Samples were seeded with 1×10^5 cell/mL and counted at each time point using the same MTT assay described above. HUVECs (1×10^6 cells/mL) were mixed with sterile GelMA solution, and then added to the SLA bioprinting platform for photocuring. After 3D bioprinting, a qualitative viability assay was performed using a Live-Dead assay kit for 1, 3 and 7 days of culture. At predesigned day post-encapsulation, the cell/hydrogel complexes were treated with calcein AM (2 μM) and propidium iodide (4 μM). Samples were observed and imaged using a Zeiss 710 confocal microscope. The HUVEC proliferation was measured to investigate cell activity when cells (1×10^6 cells/mL) were encapsulated in hydrogel. After the predetermined period, the incubation medium was changed with alamar blue assay solution (10% v/v in medium) (Invitrogen). After 4 h of incubation, the absorbance values of supernatant solution were measured at 570 and 600 nm on photometric plate reader. hMSCs encapsulated in the hydrogel also underwent the same viability and proliferation studies using the same conditions.

Construction of Vascularized Bone Grafts: For visualized vascularized bone grafts, the fluorescent labeled cell study was conducted on aforementioned 3D scaffolds. Before cell

seeding, hMSCs and HUVECs were incubated with CMFDA and CMTMR (10 μM Molecular Probes, CellTracker™ Dye, life technologies) for 30 min each at 37 °C, respectively. First, hMSCs stained with red dye (2×10^5 cells/mL) were seeded onto hard scaffolds for 24 h to ensure cell adhesion. According to the previous study, a 1:1 ratio was optimally chosen in co-culture studies as it provided robust and stable vascular networks.[2] HUVECs (green) (1×10^6 cells/mL) were mixed with GelMA solution with a 1:1 ratio and then photocured by SLA bioprinting to form cell-laden hydrogels. The cell location or arrangement in the 3D bioprinted vascularized bone constructs was imaged with confocal microscope. To investigate the effect of the scaffold's surface features on the hMSC spreading and hydrogel encapsulation for HUVEC phenotype, the organization of actin filaments was evaluated after cells cultured on our constructs for 3 d. The cells' cytoskeleton was identified with double staining of actin (red) using Texas Red labeled phalloidin and nuclei (blue) using 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen). Cells were fixed in 10% formalin for 15 min, permeabilized in 0.1% Triton X-100, and blocked with 1% BSA. Cells were then incubated with phalloidin for 20 min and DAPI for 3 min. Samples were observed and imaged using a confocal microscope.

Dynamic Culture in Bioreactor Device: In order to mimic surrounding fluid present *in vivo*, a custom-designed flow bioreactor system was utilized for incubating cells on 3D bioprinted constructs to study vascularized bone formation in dynamic culture. The culture medium was perfused through constructs using a digital peristaltic pump (Masterflex, Cole-Parmer) at a flow rate of 5 mL/min over the whole experiment period. A fluid reservoir provided the culture medium for circulation and a port for gas exchange with 5% CO_2/95% air. Efficient transfer of nutrients and oxygen is facilitated by the convective forces provided by creep flow as the medium flows through the cell/scaffold constructs. Static culture served as the control and was identically operated in the tissue culture wells. To perform this dynamic development

in our constructs, representative hydrodynamic parameters specifically permeability (K_D) were calculated using Darcy's Law.

$$K_D = \frac{\mu Q L}{A \Delta P}$$

(Equation. 1),

where μ is the viscosity of the perfusing fluid (culture medium, 8×10^{-4} Pa·s at 37 °C), Q is volumetric flow rate (cm^3/s), L is the thickness of the sample (cm), A is the cross-sectional area (cm^2), and ΔP is the pressure gradient (In our constructs, hydrogel region 230 ± 65 Pa).

The average shear stress (τ) applied over the cell surface cultured in constructs was calculated by the modified Brinkman equation.

$$\tau = \frac{B \mu Q}{A \sqrt{K_D}}$$

(Equation. 2),

where τ is the average shear stress on over the cell surface (dyn/cm^2), and B is the Brinkman constant for flow around cells (B= 3/π for spheres).

In Vitro Capillary-like Networks and Vascular Lumen-like Channels Formation: To investigate the capability of vascular formation in the soft segment of our constructs for 1 w, GelMA hydrogels with hMSCs and HUVECs were printed by SLA bioprinter. HUVECs were stained with CMTMR and hMSCs were co-encapsulated into the hydrogel using the same culture and staining protocol described above. After 3 and 6 days of culture, capillary-like networks and lumen-like channels were visualized by red fluorescence using the confocal microscope. The total capillary-like length and the number of capillary-like branch points were quantified on 3D projected confocal images.

Vascularized Bone Formation: To induce vascularized bone formation, the vascularized bone grafts, co-cultured with hMSCs and HUVECs using the same protocol described above, were

divided into two culture condition groups in the bioreactor: static co-culture and dynamic co-culture. The optimal culture condition is to utilize EGM for 1 w and then a mixed medium composed by EGM and OM at 1:1 ratio for 3 weeks.[2] At predesigned time points, cells were digested in lysed buffer via ultrasonic method and freeze-melt method. The lysate was collected by centrifuge to test alkaline phosphatase (ALP) activity, collagen type I (Col I) and VEGF secretion. The ALP activity was determined for 7 and 14 days using ALP assay kit (Bioassay Systems) after the initiation of hMSC osteogenic differentiation. ALP substrate was added to the digested suspension in the dark for 30 min, and then the absorbance was read at 405 nm. Measurements were compared to p-nitrophenol standards and normalized to total cell protein. The Col I and VEGF were determined by Col I ELISA Kit (TSZ ELISA) and VEGF ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions, respectively. To assay calcium deposition or mineralization nodules on the scaffolds, a calcium detection kit (Pointe Scientific) was used to quantify the calcium deposition. The calcium deposition on scaffolds was dissolved in 0.6 M HCl, and reacted with dye reagent. Samples were read at 570 nm wavelength, and the contents were calculated with $CaCl_2$ standards. For immunostaining of capillary-like network, cell-laden hydrogel segments were fixed in 10% formalin for 15 min, and permeabilized with Triton X-100 (0.1%) in PBS for 15 min. The hydrogels were then blocked with bovine serum albumin (BSA) for 1 h, followed by overnight incubation with primary antibodies (Anti-CD31 antibody, abcam). The hydrogels were incubated with chicken anti-mouse IgG-TR secondary antibodies (Santa Cruz Biotechnology) overnight. Finally, the hydrogels were stained with DAPI, and imaged using a confocal microscope. For immunofluorescence staining of vascularized bone, the cells were fixed with 10% formalin for 15 min, permeabilized in 0.1% Triton X-100 for 15 min and blocked in 10% BSA for 30 min. Then cells were incubated with primary antibodies at 4 °C overnight. The following primary antibodies were used for staining: goat polyclonal anti-von Willebrand factor (vWF) antibodies (Santa Cruz Biotechnology) and mouse monoclonal anti-

osteopontin (OPN) antibodies (Santa Cruz Biotechnology). After incubation with primary antibodies, donkey anti-goat IgG-FITC (Santa Cruz Biotechnology) and chicken anti-mouse IgG-TR as secondary antibodies were added and incubated 1 h, respectively. Fluorescence images were observed using a confocal microscope.

Statistical Analysis: The data are presented as the mean ± SD (standard deviation). A one-way analysis of variance (ANOVA) with Student's t-test was used to verify statistically significant differences among groups, with $p < 0.05$ being statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

[1] Y. C. Chen, R. Z. Lin, H. Qi, Y. Yang, H. Bae, J. M. Melero-Martin, A. Khademhosseini, *Adv. Funct. Mater.* 2012, 22, 2027.
[2] H. Cui, W. Zhu, B. Holmes, L. G. Zhang, *Advanced Science* 2016, 10.1002/advs.201600058.

Supplemental Figures

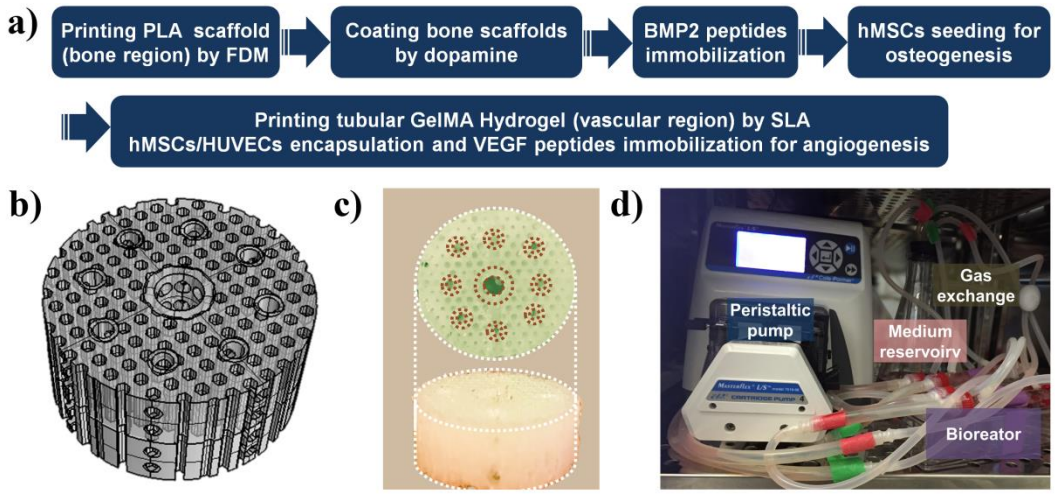

Figure S1. a) Schematic of the manufacturing process for the vascularized bone construct. b) CAD model of biphasic vascularized bone construct, including bone region and vascular channels. c) Macroscopic photo image of manufactured vascularized bone construct. Red circles show tubular vascular hydrogel regions. d) Custom-designed flow bioreactor system.

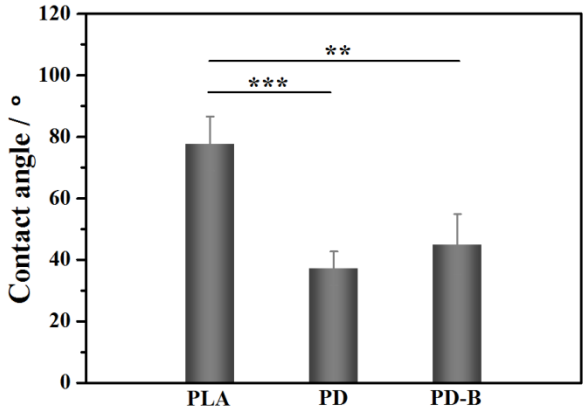

Figure S2. Contact angle measurement of different scaffold materials.

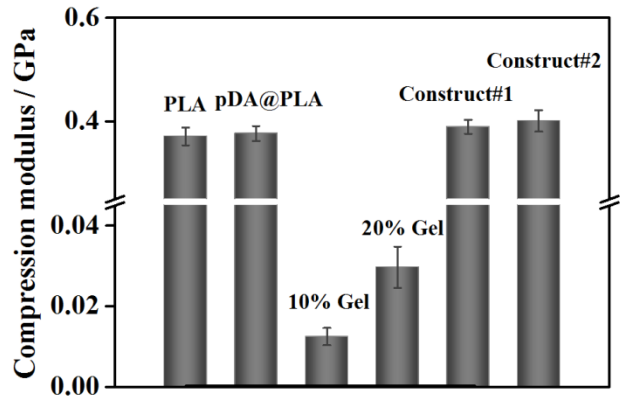

Figure S3. Mechanical properties of 3D bioprinted scaffolds, bulk hydrogels and constructs. After the fabrication, constructs maintained native bone-like mechanical strength.

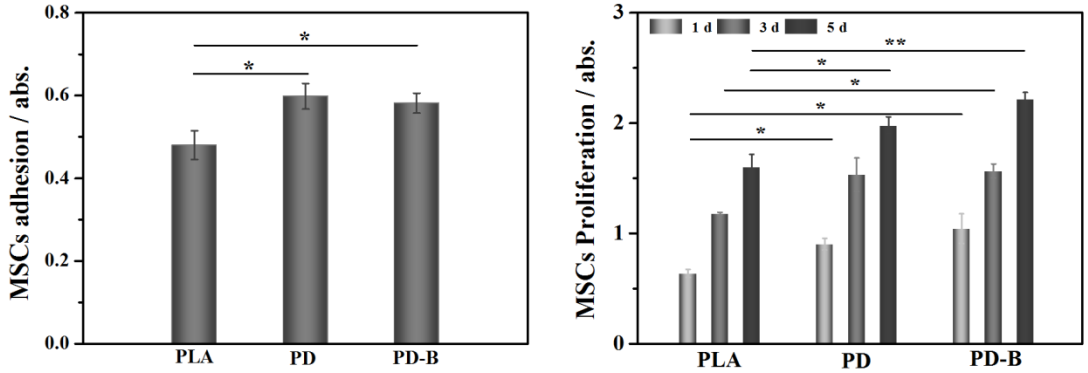

Figure S4. hMSCs adhesion and proliferation study seeded on different scaffolds.

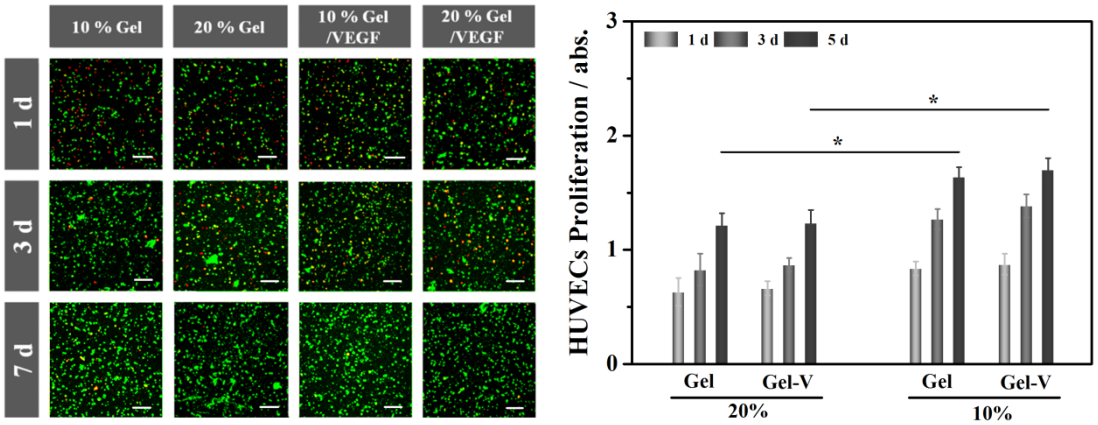

Figure S5. Live/Dead staining and proliferation of HUVECs encapsulated in 3D bioprinted hydrogels with different concentration.

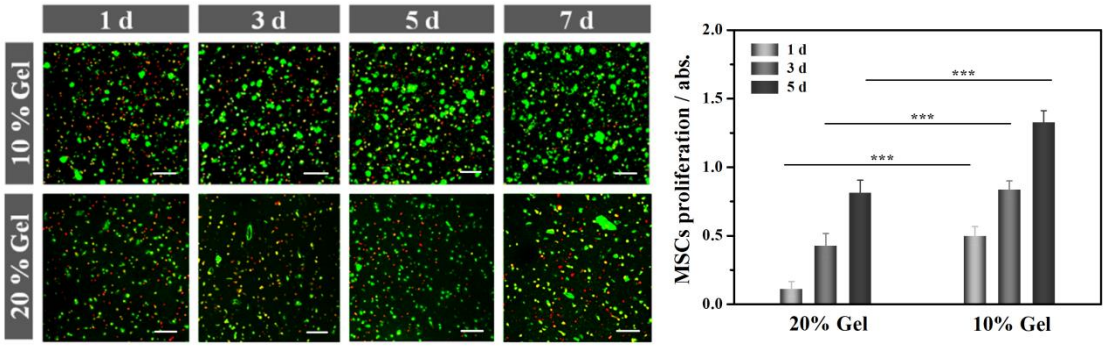

Figure S6. Live/Dead staining and proliferation of hMSCs encapsulated in 3D bioprinted hydrogels with different concentration.