ADVANCED MATERIALS

Supporting Information

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Digitally Tunable Microfluidic Bioprinting of Multilayered Cannular Tissues

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Supplementary Information

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Materials and Methods

Materials

Gelatin from porcine skin (type-A, 300 bloom), methacrylic anhydride, sodium alginate (M_w 33 kDa; low viscosity), 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959), $CaCl_2$, ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 8-arm poly(ethylene glycol) acrylate with a tripentaerythritol core (PEGOA, M_w 20 kDa) was obtained from JenKem Technology (Beijing, China). Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's modified Eagle medium (DMEM), trypsin-EDTA, 2- [4-(2-hydroxyethyl) piperazin-1-yl]-ethane sulfonic acid (HEPES buffer, 25 mM, pH 7.4), penicillin/streptomycin, DAPI, Live/dead® Viability/Cytotoxicity Kit, and PrestoBlue® Cell Viability Reagent were from Life Technologies (Carlsbad, CA, USA). Endothelial growth medium (EGM-2) and the smooth muscle growth media-2 (SmGM-2) were obtained from Lonza (Walkersville, MD, USA). Rabbit anti-human CD31, rabbit anti-mouse von Willebrand factor (vWF), rabbit anti-mouse VE-Cadherin, mouse anti-α-smooth muscle actin, rabbit anti-mouse collagen type I, and rabbit anti-mouse smooth muscle MHC antibodies, and Alexa Fluor® 594- and 488-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies were purchased from Abcam (Cambridge, MA, USA). All other chemicals used in this study were obtained from Sigma-Aldrich unless otherwise mentioned.

Synthesis of GelMA

GelMA was synthesized following a previously described protocol.[1,2] Briefly, 10.0 g of type A gelatin from porcine skin was added into 100 mL of DPBS and dissolved at 60 °C by using magnetic stirrer. Under continuous stirring, 8.0 mL of methacrylic anhydride was added drop wise to the gelatin solution and reaction was continued for 3 h at 50 °C. The reaction was

then quenched by a 5-fold dilution of the reaction mixture with warm DPBS (40 °C). The reaction product was dialyzed against distilled water at 40 °C for 7 days using 12–14 kDa cutoff dialysis tubing to remove unreacted methacrylic anhydride. Finally, GelMA solution was lyophilized resulting in a white porous foam, which was stored at room temperature until further use.

Mechanical and Rheological Properties

Mechanical properties of the bioinks with varied concentrations (w/v) of GelMA (5 and 7 %), sodium alginate (2 and 3 %), and PEGOA (1 and 2 %) in buffer medium (10 % (v/v) FBS and 25 mM HEPES in distilled water) along with 0.25 % (w/v) photoinitiator were studied. A cylindrical polydimethylsiloxane (PDMS) mold (8 mm in diameter and 2 mm in height) was filled with each of the prepared bioink compositions and crosslinked with 3% $CaCl_2$ in 10% (v/v) FBS followed by exposing to UV light (wavelength 360–480 nm, power 6.9 mW cm^{-2}) for 30 s at a distance of 8 cm from the sample. The hydrogels were then detached from mold and washed with 20-mM EDTA solution for 5 min. The compressive strengths of hydrogels were then tested using an Instron™ mechanical testing machine (Model 5542, Norwood, MA, USA), at a rate of 1 mm min^{-1} and a 60% strain level. Bluehill software was used to record the compression (mm) and load (N) and then compressive modulus was determined by taking the slope in the linear region of the stress-strain curve at 0–10% strain.

The rheological properties of different bioink formulations were analyzed at room temperature using RHEOPLUS-32 rheometer provided with cone-plate geometry (Anton Paar, Stuttgart, Germany). Each composition was analyzed by running a shear rate ramp from 1 s^{-1} to 1000 s^{-1} shear rate over the period of 10 min.

Multiple Channel Coaxial System

The extrusion-based multiple channel coaxial bioprinting system mainly included three

parts: microfluidic syringe pumps, a NovoGen MMX Bioprinter™ (Organovo, San Diego, CA, USA) with assembled nozzle device, and the computer-aided design and control system. A coaxial nozzle device with multiple injection channels was fabricated using different size needles, a 27G needle in the core, a 19G needle in the middle layer, and a 14G needle in the outer shell layer (BD Biosciences, San Jose, CA, USA). The needles were concentrically fixed using 5-minute epoxy (Devcon, MA, USA). Three independent syringe pumps (Harvard Apparatus) were used to control extrusion of bioinks at speeds of 250 μL min^{-1}, 15 μL min^{-1}, and 25 μL min^{-1}. The bioprinting processes were controlled by computer-assisted custom-coded programs.

Cell Culture

HUVECs, hSMCs, NIH/3T3 fibroblasts, and C2C12 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HUVECs and hSMCs were cultured in EGM-2 and SmGM-2, respectively. HUCs and HBdSMCs were purchased from ScienCell. HUCs were maintained in urothelial cell medium supplemented with 1% penicillin/streptomycin and urothelial cell growth supplement. HBdSMCs were maintained in smooth muscle cell media. All other cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines, seeded in tissue culture polystyrene flasks, were incubated at 37 ºC in 5% CO_2 cell. incubator. The medium was replaced every 2–3 days. The cells were sub-cultured when they reached approximately 80–90% confluency. Based on our previous study,[3] a combined medium consisting of 1:1 volume ratio of EGM-2 and SmGM-2 was used for bioprinting of hSMCs/HUVECs and for subsequent experiments.

Bioprinting

Various concentrations of GelMA (5 and 7%, w/v), alginate (2 and 3%, w/v), and PEGOA (1 and 2%, w/v) were dissolved in buffer medium (10% (v/v) FBS and 25-mM HEPES in

distilled water. 0.25% (w/v) UV photoinitiator, Irgacure 2959, was then added to blend the bioink to induce photocrosslinking. 3% (w/v) $CaCl_2$ in 10% (v/v) FBS was used as an ionic crosslinking agent and 20-mM EDTA in PBS was used as a chelator for alginate. When necessary, solutions were sterilized by filtration through a sterile 0.22-μm filter, and stored at 37 ºC incubator until printing. EDTA was stored at 4 ºC until further use.

The multiple channel coaxial bioprinting system was used to extrude 3D tubular structures by a computer-assisted software. The bioprinted constructs were first crosslinked with 3 % $CaCl_2$ in 10 % (v/v) FBS followed by exposing to UV light (360–480 nm, 6.9-mW cm^{-2} power) for 30 s at a distance of 8 cm distance from the sample. The bioprinted tubular constructs were examined using a Zeiss AxioObserver inverted fluorescence microscope (Zeiss, Thornwood, NY, USA). Similarly, tunable circumferentially multilayered tissues were bioprinted continuously using custom-coded programs and by controlling the flow rate of injecting channels. The perfusability of bioprinted tube was demonstrated by injecting fluorescent dyes into the bioprinted tube using a syringe pump. Images and videos were recorded at different time points.

For bioprinting 3D tubular tissues, the cells were mixed gently with prepolymer bioink just before bioprinting. The bioprinted tissue constructs were immediately immersed in 3 % (w/v) $CaCl_2$ solution for ionic crosslinking and then exposed to UV light for covalent crosslinking, under similar conditions as described above. Finally, to remove alginate, the constructs were treated with 20-mM EDTA. Afterwards, the bioprinted tissues were transferred into 6-well plates with respective medium for culture and downstream analyses.

Cell Viability and Proliferation Assay

To assess the viability of cells in the bioprinted constructs, a LIVE/DEAD® Cell Viability Kit was used according to the manufacturer's instructions from day 0 to day 7 or day 14 after

bioprinting. Briefly, bioprinted tissues were treated with calcein AM (green) and ethidium homodimer-1 (red) at 1:4 ratio in PBS, followed by incubation at 37 ºC for 15 min. After washing with DPBS, stained tissues were examined using a Zeiss AxioObserver inverted fluorescence microscope. Six images from different regions of each of the three bioprinted tissues were selected randomly for each condition to quantify the live and dead cells using ImageJ software. Finally, the cell viability was expressed as the percentage of the number of live cells to total number of cells.

Similarly, metabolic activities of cells in bioprinted tissues were analyzed using PrestoBlue® Cell Viability Reagent (10% (v/v)) at day 0–7 after bioprinting. After washing with PBS, PrestoBlue solution was added to the samples and incubated for 2 h at 37 ºC. 200 µL of the supernatant was taken from four parallel samples for each condition and optical density (OD) was measured at 570 nm using Synergy HT Microplate Reader (Biotek, Vermont, USA). The bioprinted constructs without cells were treated the same way and used as control. Data were normalized against the OD value on day 0 for each sample.

Immunocytochemistry

To demonstrate biological function of bioprinted tissues, we performed immunofluorescence staining of cell specific markers. At designed time points of culture, bioprinted tissues were fixed with 4% (w/v) paraformaldehyde for 30 min, followed by treating with 0.1% (v/v) Triton X-100 in PBS for 30 min to permeabilize the cell membrane. The samples were then blocked with 1% (w/v) BSA in DPBS for 1 h at room temperature. F-actin cytoskeleton was stained by incubating bioprinted tissues with Alex Fluor 594-phalloidin (1:40 dilution in 0.1% (w/v) BSA) at room temperature for 1 h, followed by DAPI staining (1:1000). For other antibody staining, the bioprinted tissues were incubated with primary antibodies (1:200 dilutions) at 4 ºC overnight. After washing with DPBS, the samples were incubated with

secondary antibodies (Alexa Fluor® 594- or 488-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:200 dilutions) at room temperature for 2 h. Samples were washed with DPBS and then stained with DAPI for 15 min at room temperature. Finally, the samples were examined using Zeiss AxioObserver inverted fluorescence microscope or laser scanning confocal microscope.

Statistical Analysis

All data were collected and analyzed with Statistical Package for the Social Sciences (SPSS) software (Chicago, IL, USA). All values were expressed as average ± standard deviation and a p value of <0.05 was considered to be statistically significant.

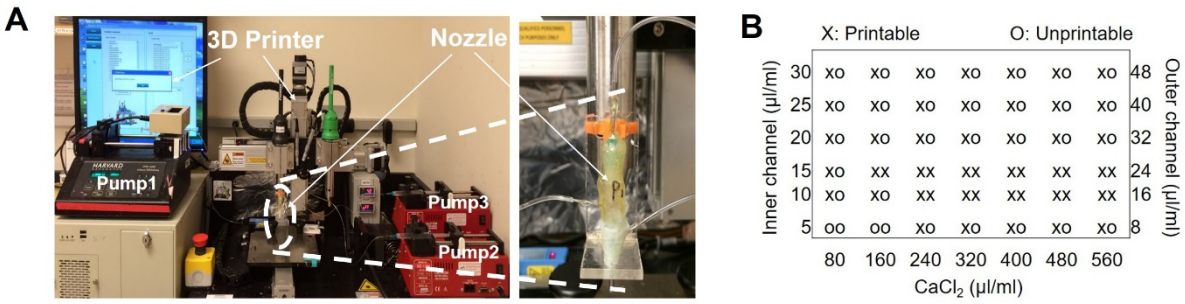

Figure S1. Multiple Channel Coaxial Extrusion System. (A) Overview of all MCCES components. (B) Printability analysis of GAP hydrogel with varying flow rate in outer and inner channels.

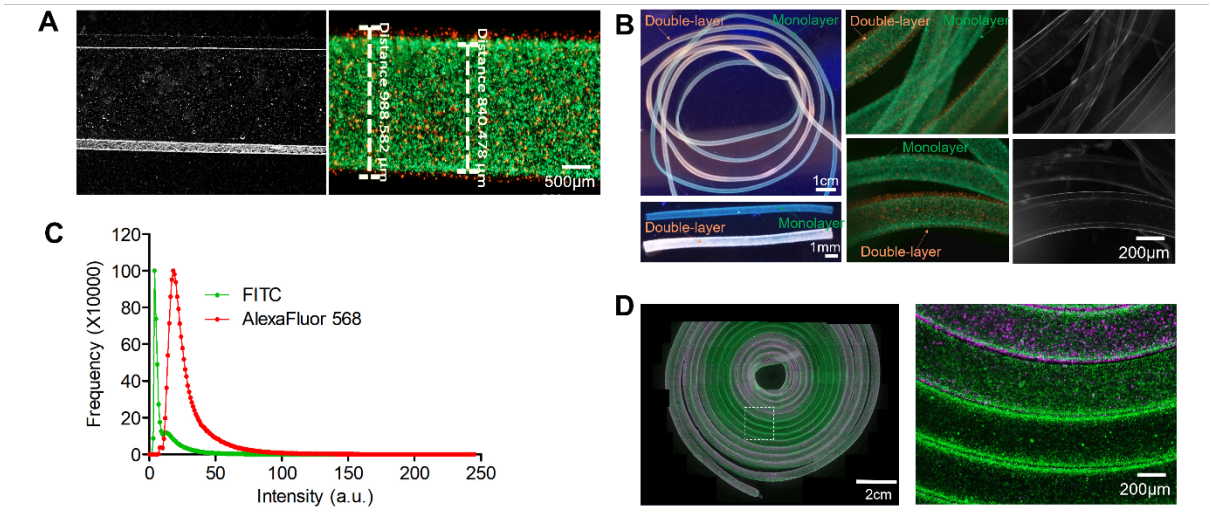

Figure S2. 3D printing of tunable and perfusable multi-circumferential layered constructs with MCCES. (A) Representative image of printed hollow fiber with loaded fluorescent microbeads (green: inner layer and red: outer layer), showing the diameters of inner and outer layers. (B) Fluorescent micrographs showing printed double-layered hollow tubes, where green fluorescent beads were embedded into inner layer and red fluorescent beads were embedded into outer layer during the printing process. (C) Intensity of red and green signals at the region of conversion of single layer to double layer. (D) Fluorescent micrographs showing dynamic conversion between single and double-layered hollow tube with clear demarcation of single and double-layered region of hollow tube.

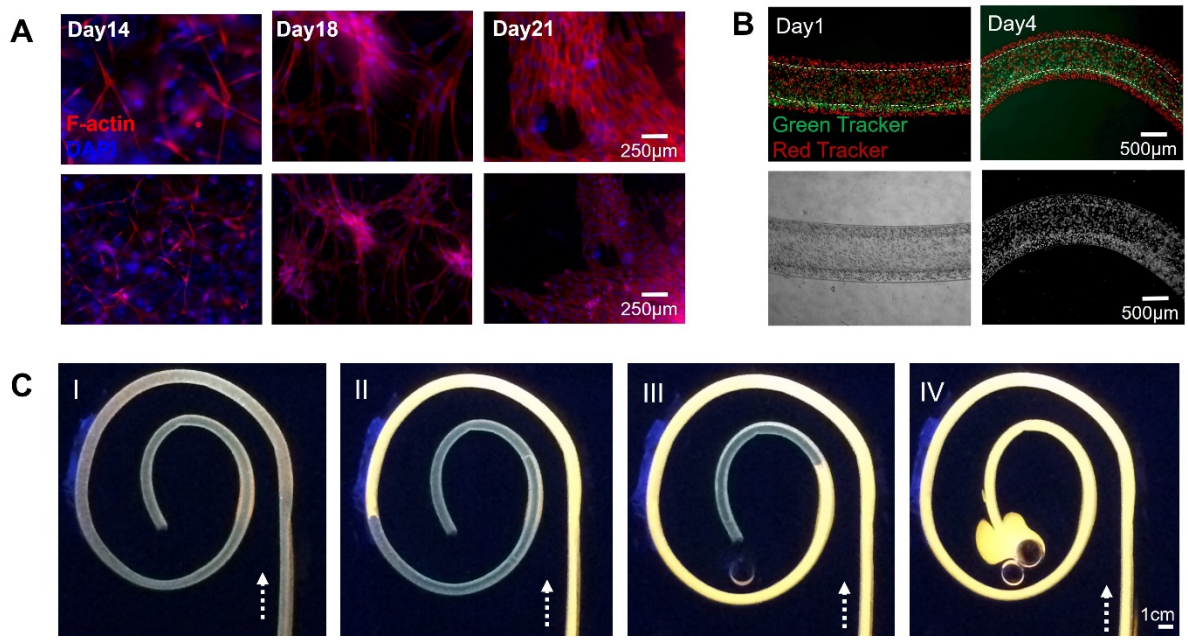

Figure S3. Biocompatibility of GAP, 3D bioprinting with MCCES and perfusion of bioprinted tube. (A) F-actin/ DAPI stained images of monocytes within GAP on day 21. (B) Fluorescent images of bioprinted inner NIH/3T3 cells labeled with green cell tracker and outer C2C12 cells labeled with red cell tracker on day 4. (C) Perfusion of NIH/3T3 cell laden hollow fibers.

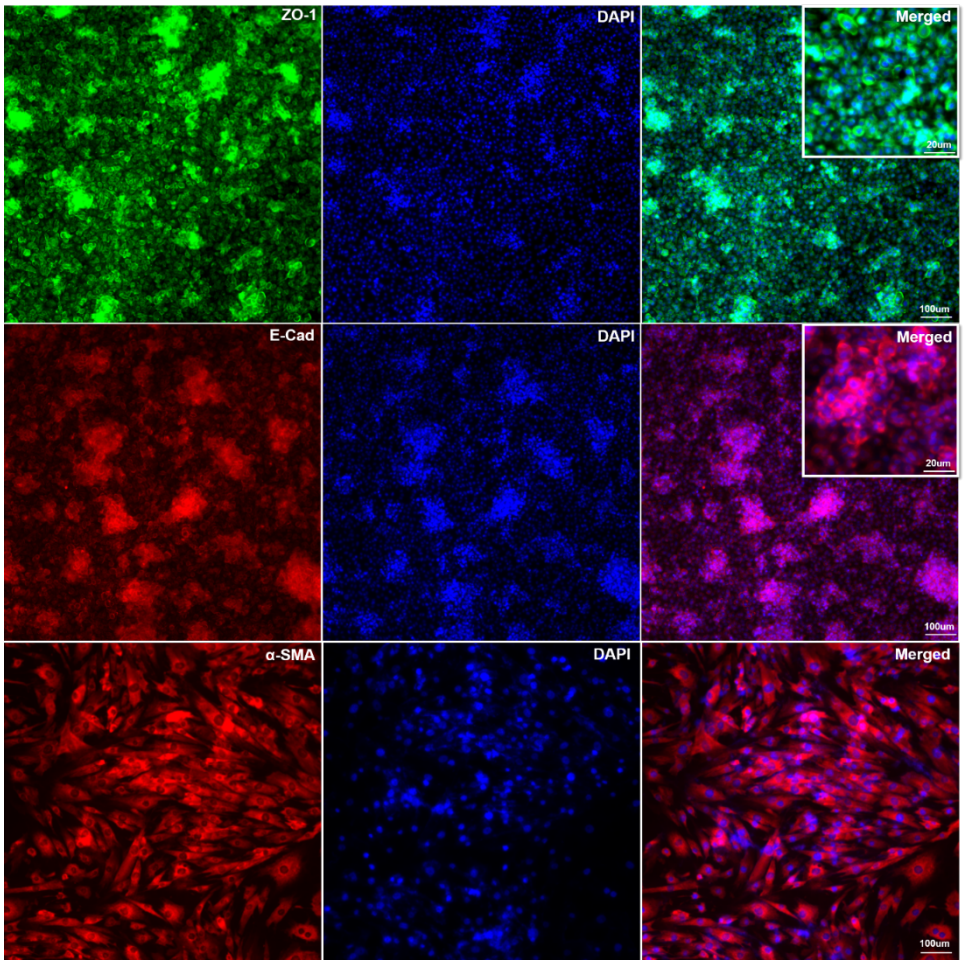

Figure S4. Biocompatibility of GAP bioink for urothelial cells. Fluorescent microscopy images of the immunostained urothelial cells encapsulated in 3D GAP scaffold after 7 days of culture showing the expression of urethral cell specific biomarkers, ZO-1 (green) and E-cadherin (red) by HUCs, and α-SMA (red) by HBdSMCs. The top insets show the respective confocal images.

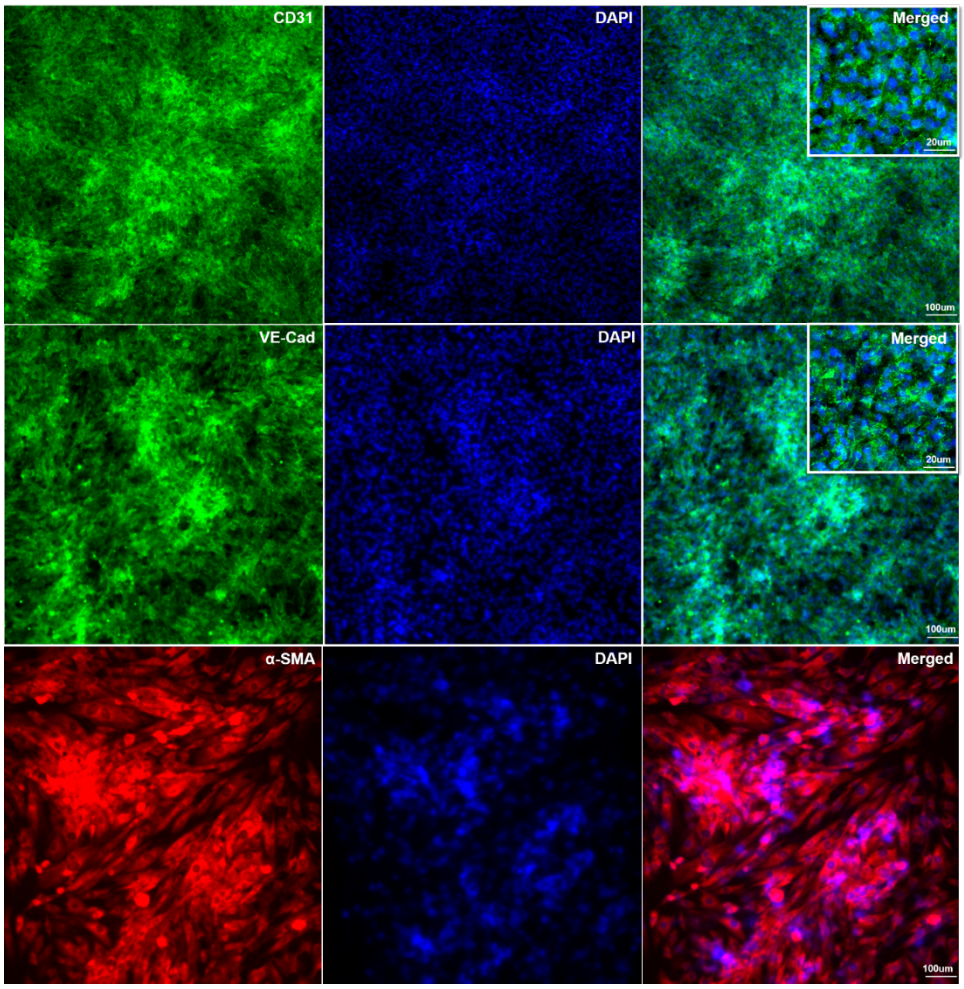

Figure S5. Biocompatibility of GAP bioink for vascular cells. Fluorescent microscopy images of the immunostained vascular cells in 3D GAP scaffold after 7 days of culture showing the expression of vascular endothelial cell specific biomarkers, CD31 (green) and VE-cadherin (green) by HUVECs, and α-SMA (red) by hSMCs. The top insets show the respective confocal images.

Movies

Movie S1. Bioprinting process with MCCES.

Movie S2. Perfusion of a bioprinted double-layer hollow fiber.

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