Supporting Information

A Versatile Strategy to Construct Free-Standing Multi-Furcated Vessels and Complicated Vascular Network in Heterogeneous Porous Scaffolds via Combination of 3D Printing and Stimuli-Responsive Hydrogels

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(1) Optimization of Gel/Chit weight ratio in Gel/Chit-H^+ solution

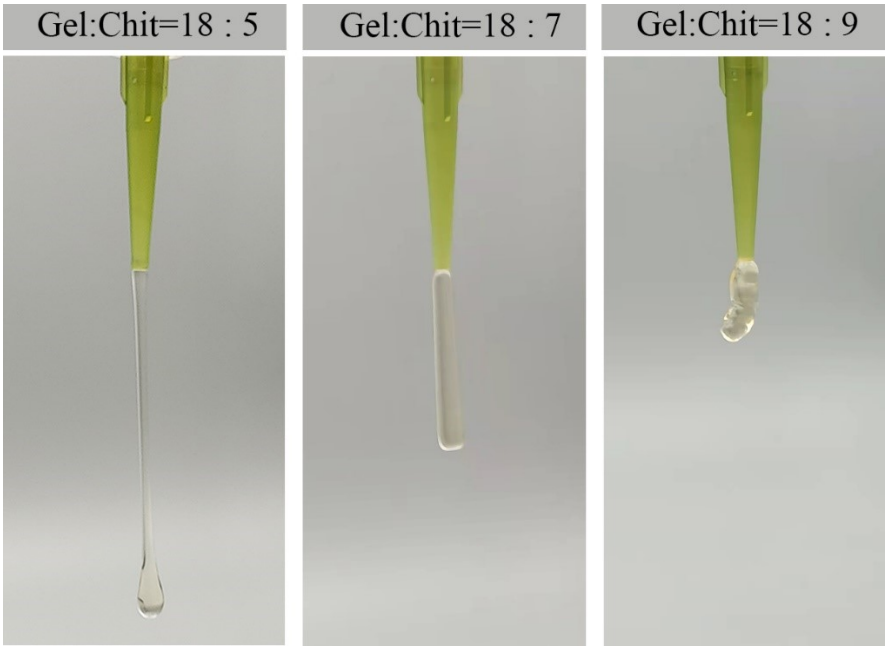


Figure S1. Optimization of Gel/Chit weight ratio by the printability of Gel/Chit-H^+ solution. The weight of chitosan was 5, 7, 9 g respectively (the total weight of chitosan and acetic acid solution is fixed at 100 g), and the weight of gelatin was fixed at 18 g.

As shown in Figure S1, when the Gel/Chit weight ratio is 18:5, Gel/Chit-H^+ solution (ink) can be extruded easily from the tapered tip, but the filament is not of uniform shape due to the low viscosity that cannot conquer its own gravity. When the Gel/Chit weight ratio is 18:7, the ink can be extruded continuously and smoothly and form a relatively long filament before dropping. However, when the Gel/Chit weight ratio is 18:9, the ink is stuck in the nozzle head and burst extruded after high-pressure buildup. As a result, the Gel/Chit weight ratio in Gel/Chit-H^+ solution is chosen as 18:7.

(2) Shear-thinning behavior of warm Gel/Chit-H^+ solution (50 ^oC)

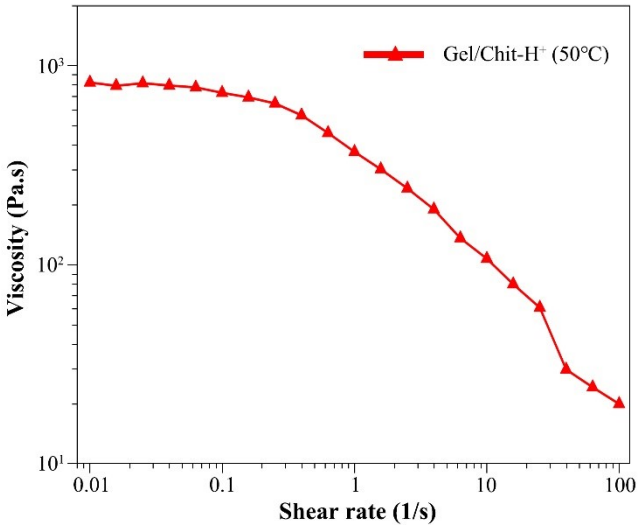
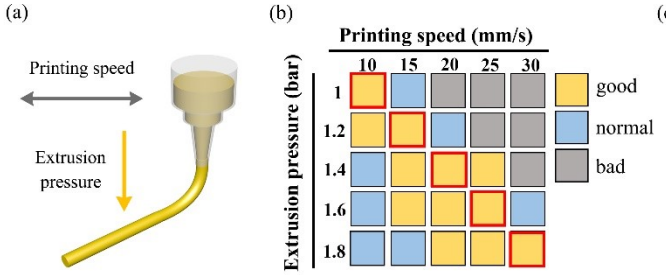


Figure S2. Shear-thinning behavior of warm Gel/Chit-H^+ solution (50 °C)

(3) Variation in outer diameter (OD) of Gel/Chit-H^+-SO_4 hydrogel tube by printing speed and extrusion pressure



(c)

Parameters	Value				
Printing speed (mm/s)	10	15	20	25	30
Extrusion pressure (bar)	1	1.2	1.4	1.6	1.8
outer diameter (mm)	5.6 ±0.15	4.8 ±0.20	4.1 ±0.15	3.7 ±0.12	3.2 ±0.21

Figure S3. Variation in outer diameter (ID) of Gel/Chit-H^+-SO_4 hydrogel tube by printing speed and extrusion pressure: (a) schematic illustration on printing speed and extrusion pressure; (b) qualitative evaluation of the printability of Gel/Chit-H^+ solution (bioink) at different printing speeds and extrusion pressures; (c) the corresponding diameters of Gel/Chit-H^+ hydrogel filaments (or namely, the outer diameters of Gel/Chit-H^+-SO_4 hydrogel tube).

(4) The formation process of bi-furcated Gel/Chit-H^+-SO_4 hydrogel tube

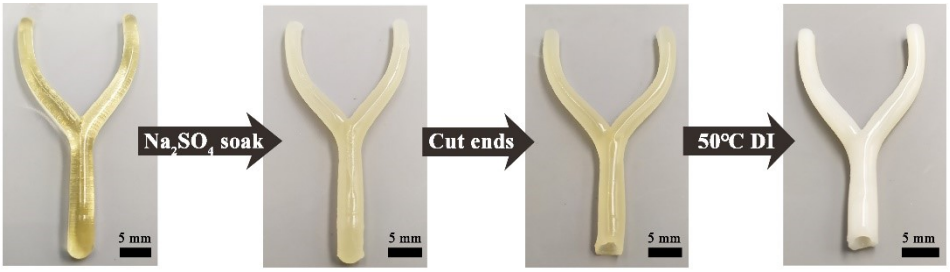


Figure S4. The formation process of bi-furcated Gel/Chit-H^+-SO_4 tube

(5) Micro-CT images of 3D-structured multi-furcated Gel/Chit-H^+-SO_4 hydrogel tubes

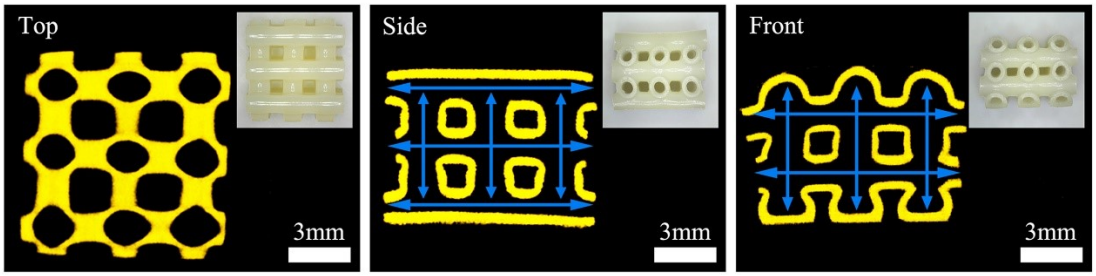


Figure S5. Micro-CT images of 3D-structured multi-furcated Gel/Chit-H^+-SO_4 hydrogel tubes

(6) Degradation rate of Gel/Chit-H^+-SO_4 hydrogel tube in PBS

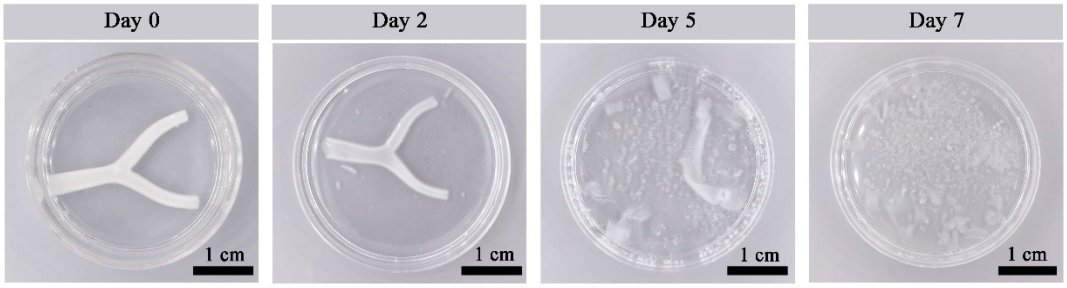


Figure S6. Degradation rate of Gel/Chit-H^+-SO_4 hydrogel tube in PBS containing lysozyme

(7) Elasticity and flexibility characterization of Gel/Chit^0 MFV

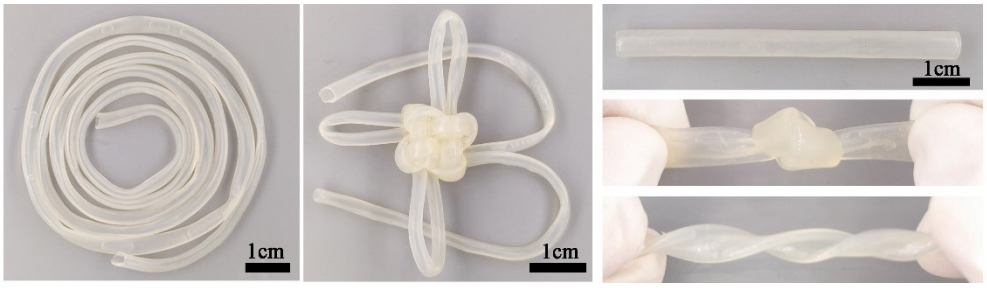


Figure S7. Elasticity and flexibility characterization by knotting and twisting Gel/Chit^0 MFV

(8) In vitro thrombosis test

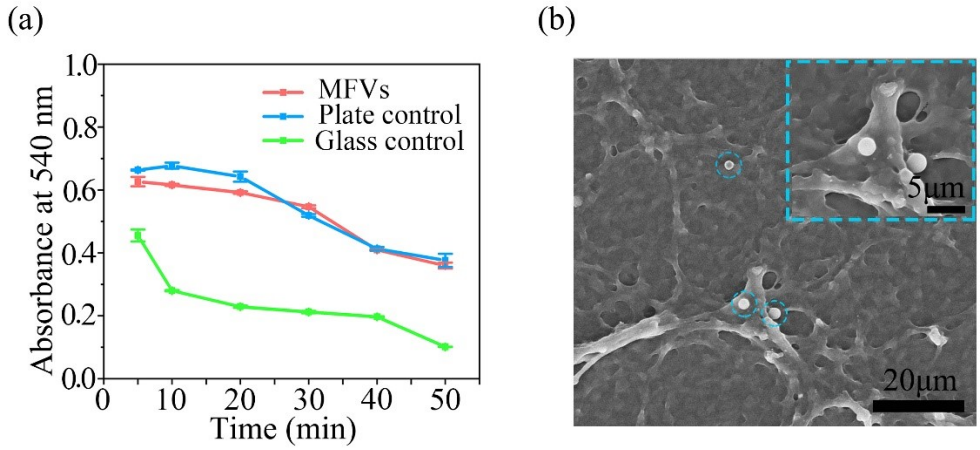


Figure S8. In vitro thrombosis test: (a) the changes in absorbance of supernatant over time when blood is in contact with the MFV, polyvinyl chloride plate (negative control) and glass surfaces (positive control); (b) SEM image of the MFV surface after incubating in platelet rich plasma for 2 h.

Whole blood coagulation time is often used as a direct reflection of blood coagulation. Glass coverslips strongly initiate blood clotting while polyvinyl chloride plates do not trigger blood clotting, which were used as positive and negative controls respectively. After dropping recalcified blood onto the sample surface and incubating for a period of time (5, 10, 20, 30, 40 and 50 min), deionized water was used to lyse the uncoagulated erythrocytes and release hemoglobin. The concentration of free hemoglobin in supernatant was quantified by measuring its absorbance at 540 nm (Figure S8a). When blood coagulation occurs, the absorbance value of supernatant will decrease rapidly. Figure S8a shows the changes in absorbance over time when blood is in contact with the MFV, polyvinyl chloride plate (negative control) and glass surfaces (positive control). As a coagulation-triggering surface, glass surface exhibits rapid blood coagulation after exposing to blood for 5 min. In contrast, MFV shows a much slower coagulation rate, similar to that of the negative group, demonstrating that MFV does not cause obvious thrombogenicity. In addition, platelet adhesion and aggregation on MFV surface were observed by SEM after incubating in platelet rich plasma (PRP) for 2h. Only a small amount of discoid-shape platelets is observed on MFV surface (Figure S8b), further confirming the non-activated state of platelets and thus non-thrombotic activity of MFVs.

(9) In vitro whole blood circulation test

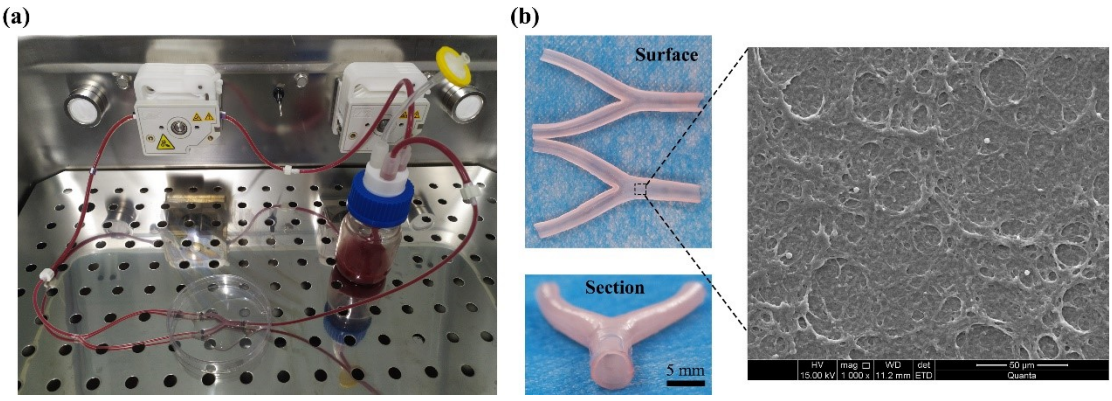


Figure S9. In vitro whole blood circulation test for 24 h at 37 °C. (a) photo of the whole blood circulation equipment; (b) images of MFV after whole blood circulation test and SEM image of the MFV surface after whole blood circulation test.

(10) Inflammation and foreign-body response of PEGDA hydrogel evaluated by rat subcutaneous implantation

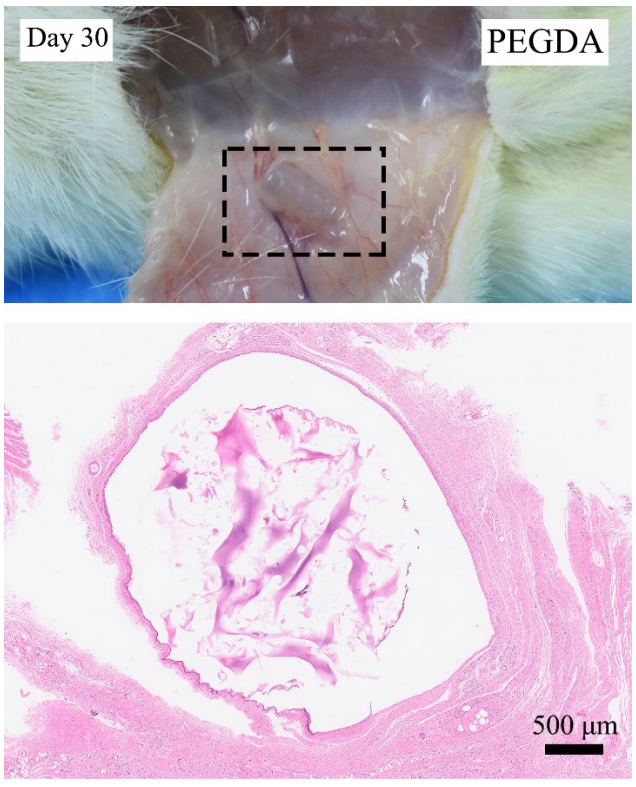


Figure S10. Inflammation and foreign-body responses of PEGDA hydrogel evaluated by rat subcutaneous implantation： (top) photo images and (bottom) HE staining of PEGDA implanted subcutaneously for 30 days.

(11) Penetration of Gel/Chit^0 hydrogel into the porous scaffold with various filling degrees

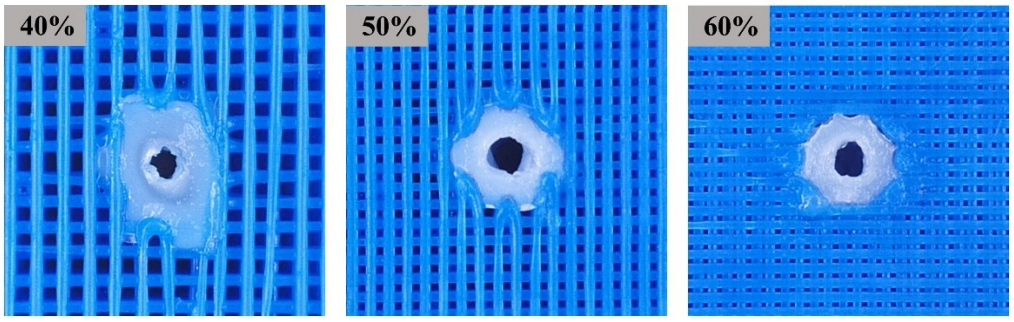


Figure S11. Penetration of Gel/Chit^0 hydrogel into the porous scaffold with various filling degrees

(12) Adjustment of Gel/Chit^0 hydrogel tube thickness via the ionic cross-linking time

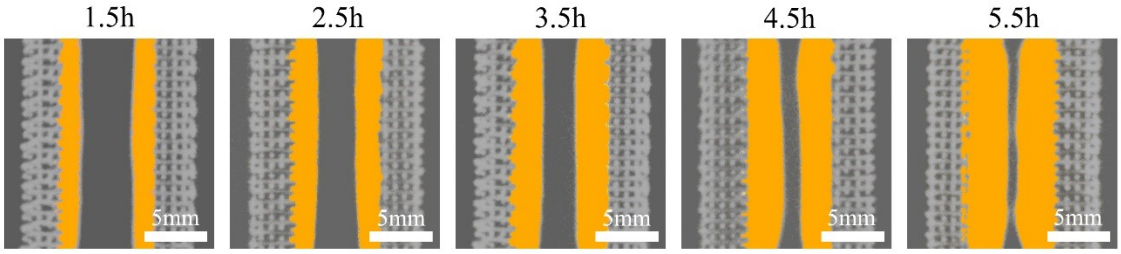


Figure S12. Adjustment of Gel/Chit^0 hydrogel tube thickness in HPS-MFV via the ionic cross-linking time

(13) Heterogeneous porous β-TCP and Gel/Chit^0 scaffold containing multi-furcated vessel (HPS-MFV)

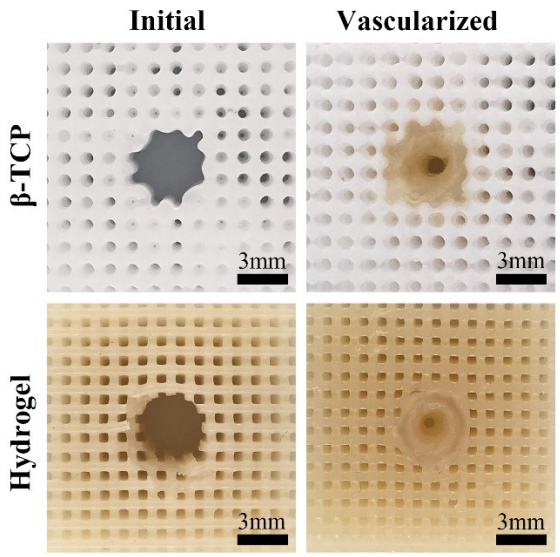


Figure S13. Heterogeneous porous β-TCP and Gel/Chit^0 scaffold containing multi-furcated vessel (HPS-MFV)

(14) Cell proliferation behaviors in HPS-MFV

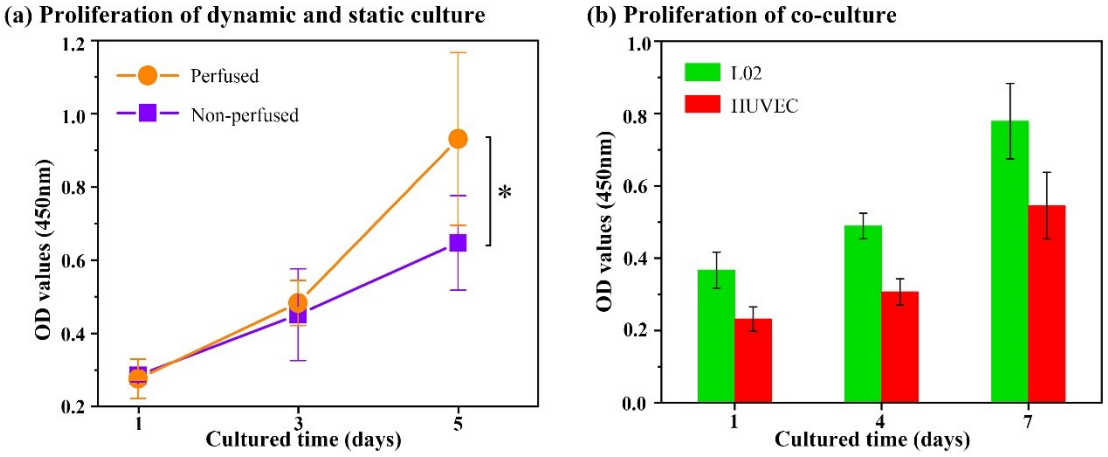
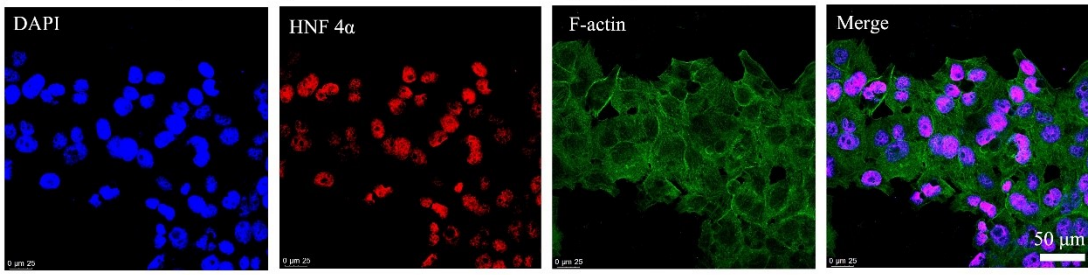


Figure S14. (a) L02 cell proliferation in HPS-MFV with or without perfusing culture medium through vessel lumen; (b) proliferation behaviors of L02 and HUVEC cells after co-culturing them in HPS-MFV.

(15) Characteristic protein expression of human liver hepatocytes and HUVECs on HPS-MFV

(a) Characteristic protein expression of human liver hepatocytes



(b) Characteristic protein expression of HUVECs

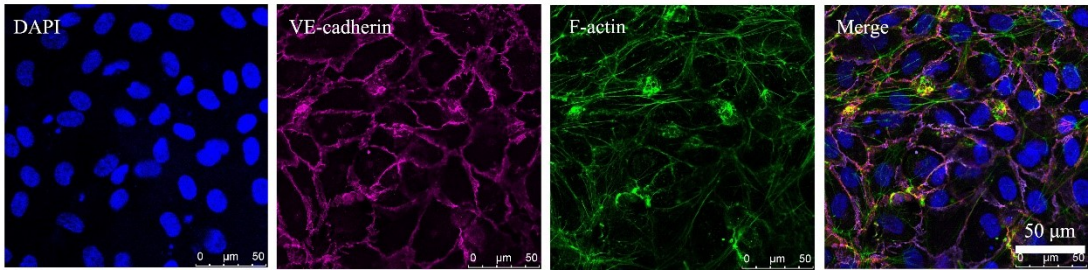


Figure S15. (a) characteristic protein (HNF4α) expression of human liver hepatocytes; (b) characteristic protein (VE-cadherin) expression of HUVECs.

Experimental section

Materials

Gelatin, sodium sulfate (purity: 99.5%) and sodium hydroxide (purity: 96%) were purchased from Aladdin (China). Chitosan (deacetylation: 95%) and acetic acid were bought from Macklin (China) and Guangzhou Chemical Reagent Company (China), respectively. Polylactic acid (PLA) was obtained from Lipai Company (China). Agar powder and food dye (blue) were purchased from Huankai Microbio Sci. & Tech (China) and Shanghai Dyestuffs Research Institute (China). 4, 6-Diamidino-2-phenylindole (DAPI), actin-tracker Green-488, calcein AM, propidium iodide (PI), and 4% paraformaldehyde were bought from Beyotime (China). Cell Tracker Green CMFDA and Cell Tracker CM-Dil were purchased from Yeasen Company (China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin streptomycin were purchased from Gibco (USA). Anti-CD31, anti-VE Cadherin and goat anti-rabbit IgG H&L were purchased from

Abcam (UK). HNF4α was purchased from Cell Signaling Technology (USA). Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell (USA). Human vascular smooth muscle cells (HVSMCs), human fetal hepatocyte (L02) and human liver hepatocytes were donated from School of Medicine, South China University of Technology (China).

Preparation and characterization of stimulus-responsive hydrogel

Chitosan solution was prepared by adding chitosan powders (7 g) into 3.5 % (v/v) of acetic acid solution (93 g) and stirring for 12 h at room temperature. Gelatin (18 g) was then added to the chitosan solution, stirred overnight at 50 °C and placed in an oven for 48 h to remove air bubbles. The dual-network Gel/Chit-H^+-SO_4 hydrogel was obtained by cooling the above hybrid solution at 4 °C for 1 h to form a gelatin network, and then soaking in a sodium sulfate solution (0.3 M, pH 7.0) at room temperature for 12 h to form the electrostatic network between chitosan and sulfate anion. The as-prepared hydrogel was finally immersed in deionized water for 2 days to achieve swelling equilibrium and remove excess sodium sulfate. The dual-network Gel/$Chit^0$ hydrogel was prepared by immersing Gel/Chit-H^+-SO_4 hydrogel in NaOH solution (1 M, pH 11) for 6 h.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra (Nexus Por Euro, USA) were collected to confirm the interactions in the dual-network hydrogels. Hydrogel samples were lyophilized with vacuum freeze-drying equipment (Alpha 1 2 LD plus, Martin Christ, Germany). The rheological properties of hydrogels were investigated using a strain-controlled rheometer (MCR 302, Anton Paar, Austria) with parallel plates of diameter of 25 mm. Hydrogels were formed in square molds (length 100 mm, width 100 mm, height 1 mm) and cut into round shapes (diameter 25 mm, thickness 1 mm). The compression properties of hydrogel were probed by a universal testing system (INSTRON-5967, Instron, USA) with a 500 N sensor. Hydrogel samples were formed in the 2 mL syringe (diameter 25 mm) and compressed to failure at a speed of 1 mm/min. The moduli were calculated by taking the initial slope (5%-15%) of the stress-strain curve.

Fabrication of multi-furcated vessels (MFVs) and heterogeneous porous scaffolds containing multi-furcated vessel (HPS-MFV)

MFVs were fabricated as follows. Viscous Gel/Chit-H^+ solution was kept at 50 °C for 1 h in the barrer, and then directly printed on the cooling platform (4 °C) through the extrusion nozzle (18

G) of the 3D-BioPlotter (Envision TEC, Germany) to form straight or multi-furcated hydrogel filaments according to the expected pattern. The printed Gel/Chit-H^+ hydrogel filaments were soaked in sodium sulfate solution and then heated to melt the core, allowing the formation of Gel/Chit-H^+-SO_4 hydrogel tubes. In order to obtain hydrogel tubes with different outer diameters, we changed the extrusion pressure (1.0, 1.2, 1.4, 1.6 and 1.8 bar) and printing speed (10, 15, 20, 25 and 30 mm/s), and then soaked the printed filaments in sodium sulfate solution for 8 min. The inner diameters of Gel/Chit-H^+-SO_4 hydrogel tubes were changed by varying the soaking time (10, 20, 30, 40 and 50 min) in sodium sulfate solution (printing speed: 15 mm/s, printing pressure: 1.2 bar). The tube cross sections were observed using fluorescence microscope, and the tube diameters were measured by accessary software. The as-prepared Gel/Chit-H^+-SO_4 hydrogel tubes were finally treated by NaOH to fabricate Gel/Chit^0 MFVs.

Fused deposition modeling (FDM) was chosen to create a porous polylactic acid (PLA) scaffold that reserved void space for multi-furcated channels. 9 wt% of gelatin was added into 0.3 mol/L of sodium sulfate solution for thickening. The printed PLA scaffold was then immersed in the sodium sulfate/gelatin solution (50 ^{o}C), vacuumed for 10 minutes to make the solution completely enter the microporous structure, slowly taken out and placed in the refrigerator (4 ^{o}C). Viscous Gel/Chit-H^+ solution (50 ^{o}C) was poured into the reserved channels in the porous scaffold, cooled at 4 ^{o}C, rest for a certain time, heated to melt the core and sodium sulfate/gelatin hydrogel in the porous scaffold and finally treated with NaOH solution, leading to the formation of HPS-MFV.

Physical and chemical characterization of MFVs and HPS-MFV

Degradation test: Multi-furcated tubes with and without treatment with NaOH solution were incubated in a phosphate buffer solution (PBS) mixed with lysozyme (1 g/L) at 37 ℃ for 5, 7, 14, 21 and 90 days, respectively. At each time point, photos were taken to validate the degradation degree of MFVs. PBS solution containing lysozyme was changed every 2 days.

Burst pressure test: The radial mechanical strength of hydrogel tubes was evaluated by measuring the maximum pressure sustained under water pressure (namely, burst pressure) via a home-made device. In short, a multi-way hose connector was respectively connected to the syringe, digital manometer (pressure range: 0–1MPa) and one end of the tube (the other end of the tube was

blocked). All joints were sealed with UV-cured resin to ensure air tightness. PBS was infused slowly, and the maximum pressure before hydrogel tube destruction was recorded as burst pressure.

Suture retention strength test: One end of Gel/Chit^0 hydrogel tube (length: 2 cm) was secured in the lower grip of the tensile testing device (Instron 5967), and a 7-0 suture was used to pierce through single wall of Gel/Chit^0 hydrogel tube and then connect with the upper grip. Load-displacement curves were obtained by stretching at a speed of 2 mm/min until failure. The maximum load was recorded as suture retention strength.

Hemocompatibility test: Rabbit anticoagulant whole blood was centrifuged at 3000 rpm for 10 min and the supernatant was then removed. Such procedure was repeated 2 times and the erythrocytes were redispersed in PBS (~2 wt%). For the experiment group, 1 mL of erythrocyte suspension was mixed with 4 mL of PBS, where MFVs was immersed. Meanwhile, 1 mL of erythrocyte suspension added into 4 mL of double distilled water was served as positive control group, whilst 1 mL of erythrocyte suspension added into 4 mL of PBS was served as negative control group. All the three groups were incubated at 37 ^{o}C for 2 h and centrifuged at 3000 rpm for 10 min. The absorbance was measured with a microplate reader (BioTek, USA) at 540 nm. The hemolysis rates were calculated using the following formula:

$$\text{Hemolytic rate (\%)} = (A_S - A_{nc}) / (A_{pc} - A_{nc}) \times 100\%$$

where A_S, A_{nc} and A_{pc} are the absorbance of experiment, negative control and positive control, respectively.

Semi-permeability test: The blue dye solution and erythrocyte suspension (~10 wt%) were injected into one end of the MFVs and the other ends were blocked. The whole MFV was then immersed in PBS solution, and permeability of small molecule (blue dye) and erythrocytes was recorded after 72 h.

In vitro cytobiological characterization

Cell culture and proliferation on MFVs: Human umbilical vein endothelial cells (HUVECs) and human vascular smooth muscle cells (HVSMCs) were employed respectively to characterize the cytocompatibility of MFVs. In brief, the MFVs were placed individually in 24-well plates, sterilized by soaking in 70 % ethanol overnight and washed triple times with sterile PBS before cell

culture. HUVEC suspension (2×10^5 mL^{-1}) or HVSMC suspension (1×10^5 mL^{-1}) was injected into a sterilized MFV until the MFV was fully filled with cell suspension, which was then transferred to a humidified atmosphere with 5 % CO_2 at 37 ℃. After 2h, the MFV was turned over 90°, and the cell suspension was injected again. The above operation was repeated 4 times in the lumen to make the cells attach evenly. Cell viability and proliferation measurement were performed via live/dead staining and CCK-8 assay. Live/dead dye solution was prepared by adding 1 μL of calcein AM and 2 μL of ethidium homodimer into 1 mL of PBS. The cells were then stained by live/dead dye solution at room temperature for 30 min and observed with inverted fluorescence microscope. Cell proliferation was determined after 1, 3, 5 days by CCK-8 assay. After incubation with diluted CCK-8 solution (Dojindo, Japan) at 37 ℃ for 2 h, the number of living cells was quantified by measuring the optical density of supernatant at 450 nm using a microplate reader (Thermo Scientific, USA).

Quantitative RT-PCR: Gene expressions of pro-inflammatory cytokines such as TNFα, iNOS, IL-1β and IL-6 secreted by macrophages (RAW264.7 cells) were assessed by real-time polymerase chain reaction (RT-PCR). Macrophages cultured with normal medium were used as the control group, and macrophages cultured with MFV were used as the experiment groups. The total RNA of macrophages after co-culturing for 12 and 24 h was isolated using the RNA isolation kit, according to the manufacturer's protocol. A Nanodrop reader (Thermo Fisher Scientific, USA) was adopted to detect RNA concentration and purity. Reverse transcription was implemented with a cDNA synthesis kit, following the instruction. Gene expression was quantitatively measured with SYBR-Green reagents using a QuantStudio 6 RealTime PCR System (Thermo Fisher Scientific, USA). The data were further normalized to the expression of GAPDH and quantified relative to the corresponding gene expression from the samples in control group which was standardized to 1. Gene sequences of primers and probes included housekeep gene GAPDH (Forward: 5'-CTCCCACTCTTCCACCTTCg-3'; Reverse: 5'-TTgCTgTAgCCgTATTCATT-3'), TNF-α (Forward: 5'-CTgAACTTCggggTgATCgg-3'; Reverse: 5'-ggCTTgTCACTCgAATTTTgAgA-3'), iNOS (Forward: 5'-gAgACgCACAggCAgAgg-3'; Reverse: 5'-CAggCACACgCAATgATgg-3'), IL-1β (Forward: 5'-TggAgAgTgTggATCCCAAg-3'; Reverse: 5'-ggTgCTgATgTACCAgTTgg-3'), IL-6 (Forward: 5'-ATAgTCCTTCCTACCCCAATTTCC-3'; Reverse: 5'-gATgAATTggATggTCTTggTCC-3').

Cell co-culture on HPS-MFVs: In order to distinguish the positions of different cells on the

scaffold, L02 and HUVECs were labeled respectively with live cell fluorescence tracers before culture. L02 cells were labeled with green fluorescence, while HUVECs were labeled with red fluorescence. First, L02 cells were seeded on the external porous scaffold: to prevent L02 cell suspension from flowing into the MFV lumen, a small drop of 2% agar was added to seal the tube outlet in advance. 300 μL green fluorescent-labeled L02 cell suspension with a density of 5×10^5 mL^{-1} was vertically added to the external porous scaffold of HPS-MFV and incubated for 1h. Then 500 μL of L02 suspension was slowly added from all directions to immerse the scaffold and allowed to adhere for 2 h. Thereafter, agar drops were removed from the outlet of the tube, and then red fluorescent-labeled HUVECs (2.5×10^6 mL^{-1}) were seeded into the lumen of MFV according to the method of cell culture on MFV. Finally, mixed medium (DMEM and endothelial cell medium were mixed 1:1) was added to immerse the whole scaffold, and transferred to the cell incubator for further culture.

Immunofluorescence staining: HUVEC-coated MFVs or human liver hepatocytes-coated HPS-MFV were fixed in 4% paraformaldehyde for 30 min, washed three times with PBS. Then HUVECs or human liver hepatocytes were permeabilized in 0.1% Triton-X 100 for 15 min, washed three times with PBS, treated with 3% bovine serum albumin (BSA) at room temperature for 1 h, washed three times with PBS, incubated with anti-CD31 (ab28364, 1:100), anti-VE Cadherin (ab33168, 1:2000) or HNF4α (C11F12, Rabbit mAb #3113) respectively overnight at 4 °C, washed three times with PBS, incubated with (Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor® 568) for 2 h, washed three times with PBS, stained with Actin-Tracker Green for 1 h, then stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min, observed using a confocal microscope (Leica, Leica TCS SP8) with consecutive z-stack images at ×63 (or ×40) oil mirror magnifications.

Ex vivo and in vivo characterization

Ex vivo arteriovenous shunt assay: The animal experiments were conducted in accordance with the ethical guidelines approved by the Institutional Animal Care and Use Committee of South China University of Technology. MFVs were sterilized and connected with a circulating infusion device. New Zealand rabbits (2.5 kg) were anesthetized by intravenous injection of pentobarbital sodium solution (2% (w/v), 1.5 mL/kg), and heparin (300 Unit/kg) for systemic anticoagulation. The carotid

artery and jugular vein of rabbits were isolated and connected with both ends of the infusion tube to form arteriovenous extracorporeal circuit. After 2 h of circulation, MFVs were cleaned with physiological saline, and the lumen was observed. Then the MFV was cut open, fixed in 4% paraformaldehyde, dehydrated with gradient ethanol and observed by SEM.

Blood clotting assay: 100 μL of citrated whole blood containing 0.1M Ca^{2+} was respectively dropped on the Gel/$Chit^0$ hydrogel surface, the blank polyvinyl chloride plate surface (negative control) and glass coverslip surface which caused strong blood coagulation (positive control). After periodic incubation at 37 ℃ for 5, 10, 20, 30, 40 and 50 min, 3 mL of distilled water was slowly injected and rested for 10 min to lyse the uncoagulated erythrocytes and release hemoglobin. The concentration of free hemoglobin in distilled water was quantified by measuring the absorbance of supernatant at 540 nm.

Platelet activation test: To study platelet adhesion on Gel/$Chit^0$ hydrogel, platelet-rich plasma (PRP) was obtained by centrifugation from Fresh New Zealand white rabbit blood. The hydrogels were immersed in PBS, equilibrated for 2 h, cut into circular samples with a diameter of 10 mm and placed into culture plates containing 200 μL of PRP. After incubation at 37 °C for 2 h, PRP was decanted off and the samples were gently rinsed with PBS solution to wash away nonattached platelets. Then the samples were fixed with 4% paraformaldehyde for 24 h and dehydrated with gradient alcohol (30, 50, 70, 80, 95, and 100%). Finally, platelet adhesion was observed by SEM.

Subcutaneous implantation: In vivo compatibility was assessed by subcutaneous implantation. Seven weeks old male Sprague Dawley rats (weight: 15–200 g) were anesthetized by intraperitoneal injection of pentobarbital sodium solution (1% (v/v), 3 mL/kg), At the same time, the sterilized grafts (all with 2 mm inner diameter, 5 mm length) were symmetrically implanted on both sides of rat back. After 5, 10 and 30 days, the rats were sacrificed and the grafts were taken out, fixed with 4% paraformaldehyde, embedded in paraffin, sectioned to 5 μm thickness and stained with hematoxylin and eosin (H&E) for histological analysis.

Statistical analysis

All fluorescence images were analyzed with ImageJ software. A one-way analysis of variance followed by Tukey's test for means comparison was performed to assess the level of significance using SPSS 19.0 statistics software. Results are expressed as the average standard error with

significant *P*-values (*$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).