ADVANCED MATERIALS

Supporting Information

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"Living" Microvascular Stamp for Patterning of Functional Neovessels; Orchestrated Control of Matrix Property and Geometry

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Supporting Information

Synthesis of MA and PEGDA. For the synthesis of methacrylic alginate (MA), 2-aminoethyl methacrylate was conjugated to the carboxylate group of alginate via EDC chemistry. The alginate used in this experiment (molecular weight (M_w) ~ 50,000 g/mol) was obtained by irradiating alginate rich in gluronic acid residues, (LF20/40, FMC Technologies, M_w ~ 250,000 g/mol) with a dose of 2 Mrad for 4 hours from a ^{60}Co source. The irradiated alginate was dissolved in the 0.1 M MES ((2-(N-morpholino) ethanesulfonic acid) buffer (pH 6.4, Sigma-Aldrich) at the concentration of 1.0 % (w/v). Then, 1-hydroxybenzotriazole (HOBt, Fluka), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Thermo Scientific) and 2-aminoethyl methacrylate (AEMA, Sigma Aldrich) were dissolved in the alginate solution and stirred for 18 hours. Both the molar ratio of HOBt to AEMA and the molar ratio of EDC to AEMA were kept constant at 2:1. The mixture was dialyzed extensively against deionized (DI) water for three days, while exchanging the DI water every 12 hours. The dialyzed alginate solution was lyophilized, and reconstituted to a 3 wt % stock solution. The conjugation of methacrylate groups onto the alginate was confirmed by ^{1}H-NMR (300MHz, QE300, General Electric), as previously reported. In this study, the number of methacrylates linked to a single alginate with molecular weight (M_w) of 50,000 g/mol was kept constant at 60. In parallel, poly(ethylene glycol) diacrylates (PEGDA) was synthesized via chemical reaction between poly(ethylene glycol) (PEG, Sigma Aldrich) and acryloyl chloride (Sigma Aldrich). First, PEG was dissolved in dichloromethane at the concentration of 10 wt %. Next, acryloyl chloride and triethylamine (Fisher Chemical) were dissolved in the PEG solution and stirred overnight under dry N_2 gas. The molar ratio of PEG, acryloyl chloride and triethylamine was 1:4:4. Finally, the insoluble salt (triethylamine-HCl) was filtered, and the product was precipitated by adding ice-cold ether. The crude product was dissolved into DI water and dialyzed for one day to remove unreacted starting materials and the salt, a byproduct. Then, the product was frozen at -20 ºC and lyophilized. The conjugation of acrylate groups onto PEG was confirmed by ^{1}H-NMR (300 MHz, QE300, General Electric).

Magnetic Resonance Imaging (MRI) of the Hydrogel. Spin echo multi-slice (SEMS) pulse sequence for MR imaging of the hydrogel was used to acquire resonance data, which were then converted into water density map using VNMR 6.1C software. For SEMS pulse sequence, the repetition time (T_R) of 2.5 s and the echo time (T_E) of 5 ms were used. The field of view (FOV) was 1.6 x 1.6 cm, and the image matrix was 128 x 64 pixels. The resulting water density images were processed to present the density spectrum for comparison using MATLAB (The Mathworks^{TM}). For visualization, pseudo-color was added to the images using the ImageJ software (free image analysis software from National Institutes of Health). For counting water intensity peaks, the rectangular gel picture from the image was selected, and the histogram of the image (count vs. color intensity) was taken using the ImageJ software.

Experimental set-up to examine localization of proteins diffused from the hydrogel with microchannels. The microscopic platform was prepared on a cover glass. Following the treatment with 0.1 M sodium hydroxide, the coverslip was siliconized with 3-

aminopropyltriethoxy silane (APES, Sigma) followed by modification with 0.5% glutaraldehyde (Sigma) in PBS. The 1.0 mL of pre-gel solution was prepared with 200 μL of 40 % (w/v) acrylamide (AAm, Aldrich), 132 μL of 2 % (w/v) bis-acrylamide (Bis, Aldrich), 50 μL of 10 % ammonium persulfate (APS, Aldrich) and 2 μL of N′,N′,N′,N′-tetramethylethylene-diamine (TEMED, Aldrich) in DI water. Then, the pre-gel solution was sandwiched and allowed to polymerize between the modified coverslip and unmodified coverslip of 22 mm diameter for 10 minutes at room temperature. After the polymerization, the unmodifed cover glass was removed, and the poly(acrylamide) (PAAm) gel-coated cover glass was rinsed twice. The surface of PAAm was functionalized with -NHS (-succinimidyl) groups by activating with *N*-sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate (Sulfo-SANPAH, Pierce) in the presence of UV light. UV with 252 nm wavelength was exposed twice for 7 minutes on the Sulfo-SANPAH solution on PAAm. The fluorescent Bovine Serum Albumin (rhodamine-BSA, Aldrich) as a model protein was encapsulated in the PEGDA-MA hydrogel containing microchannels, and the hydrogel was placed on the activated PAAm substrate. The BSA diffuse onto the –NHS (-succinimidyl) groups on the PAAm gel surface during incubation overnight. BSA reacts with –NHS groups to form covalent bonds, and the fluorescence pattern under the PEGDA-MA hydrogel was examined using the fluorescent microscope.

Numerical Analysis. An axisymmetric finite element model was created using Abaqus/CAE version 6.8 - 4. It was assumed that the diffusional flow effects of each microchannel had a negligible impact on the neighboring microchannels and therefore the modeling focused on an individual microchannel of varying diameter. Only the radius of the hole and the corresponding area of the tissue would be changed from one model to the next. The size of hydrogel stamp used in the model was held constant at 250 μm in width and 200 μm in height to correspond with the experimental stamp height and spacing between channels. A mesh was created with microchannel with diameters of 300, 500, 750, and 1,000 μm using 3 node axisymmetric triangles. The top surface of the model was assumed to have no diffusional flow or loss through the top of the gel and microchannel as was dictated by the experimental setup. The outer vertical surface was also assumed to have no diffusional flow in the horizontal direction outside of the control volume. The CAM membrane was modeled as being 400 μm thick with a constant concentration of zero at the lower boundary of the model. This constant concentration acted as a sink, thereby consuming the VEGF once it reached the bottom surface of the model. The diffusion calculations were governed by Fick's law of diffusion with diffusion coefficients of 200 $\mu m^2/s$, 1.0 $\mu m^2/s$, and 0.1 $\mu m^2/s$ for the media, CAM, and hydrogel stamp, respectively (1). The diffusion across the boundaries between different materials was assumed to be unobstructed. The diffusion coefficient for each of the boundaries was subsequently set as the average of the two materials involved. The stamp had a constant body flux of 3.1×10^{-14} $pg/\mu m^3\cdot s$ which represented the constant production of VEGF by the fibroblasts (Fig. 2*D*). All simulations were in micrometers, seconds, and picograms. Each of the diameters was modeled to simulate a period of 7 days.

Cell Encapsulation. NIH/3T3 cells (ATCC) were expanded and passaged at 37 °C with 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM, ATCC) supplemented by 10 % fetal

bovine serum (FBS, ATCC), and 1 % penicillin/streptomycin (ATCC). All the cells before the passage number of 10 were used in this study. Prior to encapsulation in hydrogels, cells were mixed with the pre-gel polymer solution. The cell density was kept constant at 2.0×10^6 cells/ml. The mixture of cell and pre-gel solution was exposed to the laser of SLA to activate hydrogel formation. The cell-hydrogels were incubated in DMEM supplemented by 10 % fetal bovine serum (FBS), while changing the media every two days. On days 0, 1, 3, 5, and 7, viability of cells encapsulated into the hydrogel was quantitatively evaluated using a MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (ATCC)] assay kit. The encapsulated cells were stimulated to secret growth factors by adding 0.2 mL of DMEM and 100 ng/ml of 12-O-Tetradecanoylphorbol-13-acetate (TPA, ATCC) reagent into a well of a 96-well plate which contains each cell-encapsulating gel. After incubating the cell-hydrogel construct for 24 hours, 100 µl of cell culture media was mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the proteome profiler mouse angiogenesis array (R&D Systems). The array membrane was washed to remove unbound material and streptavidin-Fluor® (430) conjugates (Invitrogen) and positively stained spots were imaged using a Phosphor Imager (Bio-Rad). In addition, on days 1, 3, and 5, the amount of VEGF secreted by cells was quantitatively evaluated by the sandwich enzyme immunoassay technique using the mouse VEGF Immunoassay kit (R&D Systems). The sample containing VEGF was pipetted into a well where a polyclonal antibody specific for mouse VEGF has been pre-coated and incubated for 2 hours. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse VEGF was added to the well. The enzyme reaction yielded a blue-colored product, and the color intensity was measured using a microplate absorbance reader (Synergy HT, Biotek). The measured value was converted to the amount of VEGF using a calibration curve. The amount of VEGF was further normalized by the number density of cells initially encapsulated into each hydrogel.

Chorioallontoic Membrane (CAM)-Based Angiogenesis Assay. The function of microvascular stamp to engineering neovessels pattern was examined by implanting the cell-hydrogel construct onto chicken chorioallontoic membrane (CAM). Following the initial incubation, a small window (1.0 × 1.0 cm) was created on top of each egg shell. Then, a freshly fabricated fibroblasts-encapsulating hydrogel disk (5×10^6 cells/ml) was implanted on top of the CAM of individual embryos. At days 0, 2, 4 and 7 after implantation, CAM images were captured using a S6E stereomicroscope (Leica) linked with D-Lux E Camera (Leica). In parallel, the fixed membrane was also embedded in paraffin and the cross-section was stained for α-smooth muscle actin (α-SMA Immunohistology Kit, Sigma-Aldrich) to count the number of mature blood vessels.

Supporting Figures

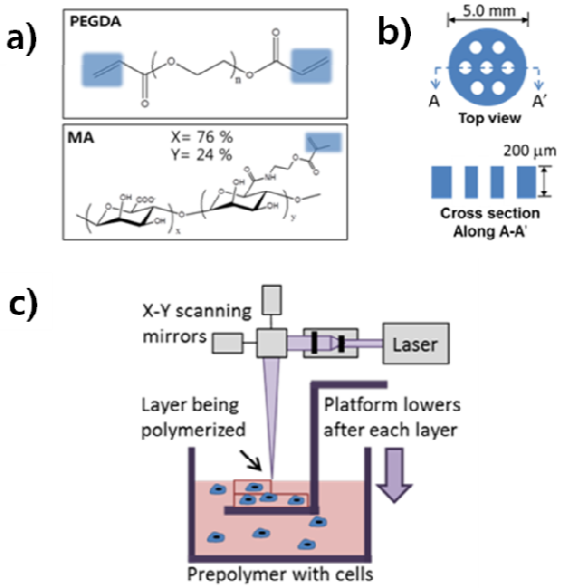

Figure S1. (a) Molecular structures of poly(ethylene glycol) (PEGDA) and methacrylic alginate (MA). (b) Schematic diagram of the stamp prepared with stereolithographic assembly. (c) Bottoms-up process for cell-encapsulated hydrogel assembly. The hydrogel with microchannels was fabricated by photo crosslinking the mixture of cell and pre-gel solution in a layer by layer fashion using a stereolithography apparatus (SLA, Model 250/50, 3D Systems). In this study, a commercially-available SLA was modified to accommodate for the bottoms-up approach as described in our previous work. In the bottoms-up approach, the pre-gel solution is pipetted into the container one layer at a time from the bottom to the top. This setup was designed to reduce total volume of photopolymer in use and also remove photopolymers used from static conditions. 3D computer-aided design (CAD) models were generated using AutoCAD 2009 (Autodesk) and exported to stereolithography (SLA) format for creating hydrogels containing microchannels. The SLA software, 3D Lightyear v1.4 (3D system), was used to slice the 3D models into a series of 2D layers from a user-specified thickness. The laser was used to selectively crosslink the pre-gel solution at a precisely calculated energy dose. The elevator controlled by the SLA was lowered by a specified distance, and the part was recoated.

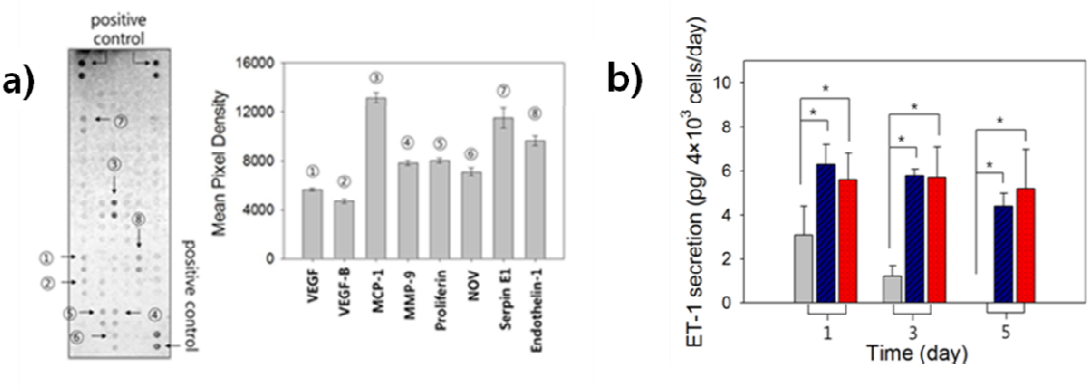

Figure S2. (a) Angiogenic growth factors including VEGF, VEGF-B, MCP-1, MMP-9, proliferin, NOV, Serpin E1 and Endothelin-1 were secreted from NIH/3T3 fibroblasts encapsulated within a PEGDA-MA hydrogel, following exposure of cells to an activator of protein kinase C. (b) On Days 1, 3, and 5, the cellular secretion level of Endothelin-1 was measured using the mouse Endothelin-1 (ET-1) Immunoassay kit (Enzo Life Sciences). The cells encapsulated within the PEGDA-MA hydrogels without microchannels (▬) and with microchannels (▬) expressed the larger amount of endothelin-1 than those within the PEGDA hydrogel (▬). The values of ET-1 secretion from the PEGDA-MA hydrogel without microchannels and from the PEGDA-MA hydrogel with microchannels were statistically different from that from the PEGDA hydrogel (*$p<0.05$).

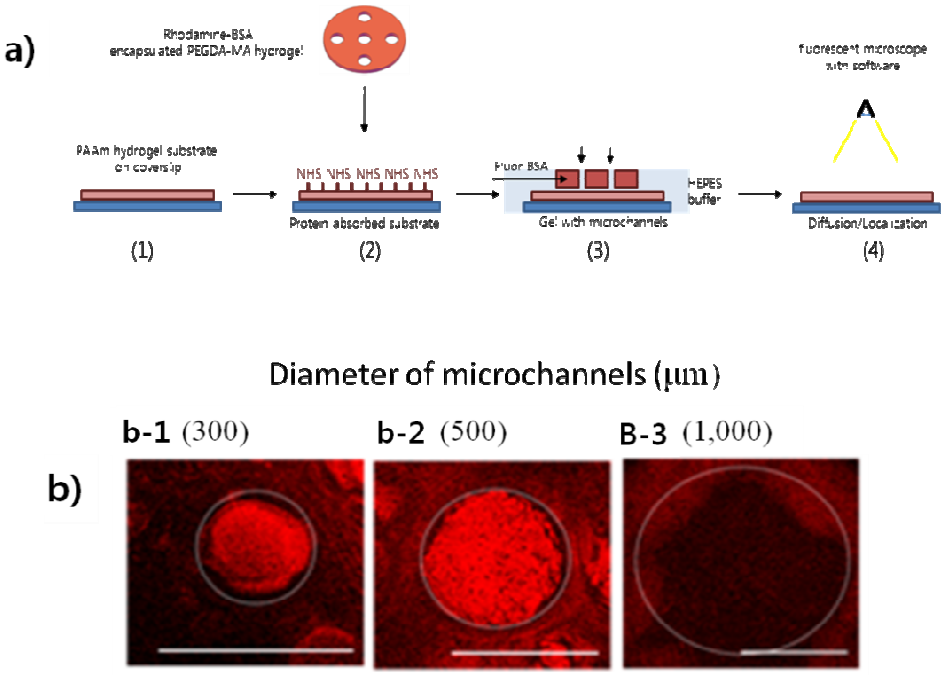

Figure S3. (a) Schematic of the experimental set-up to examine localization of proteins diffused from the hydrogel with microchannels. The poly(acrylamide) (PAAm) gel-coated microscopic platform was prepared on a cover glass (1). The surface of PAAm was functionalized with -NHS (-succinimidyl) groups (2). The hydrogel containing the fluorescent Bovine Serum Albumin (BSA) was placed on the activated PAAm substrate. The BSA diffuse onto the –NHS (-succinimidyl) groups on the PAAm gel surface during incubation overnight (3). BSA reacts with –NHS groups to form covalent bonds, and the fluorescence pattern under the PEGDA-MA hydrogel was examined using the fluorescent microscope (4). (b) The hydrogel stamp was placed on a poly(acrylamide) hydrogel modified with succinimidyl ester groups. The BSA released from the hydrogel stamps containing microchannels with diameter of 300 μm (b-1) and 500 μm (b-2) was localized within the circular pattern. In contrast, the hydrogel containing a channel with diameter of 1,000 μm showed minimal localization of BSAs within the circular pattern (b-3).

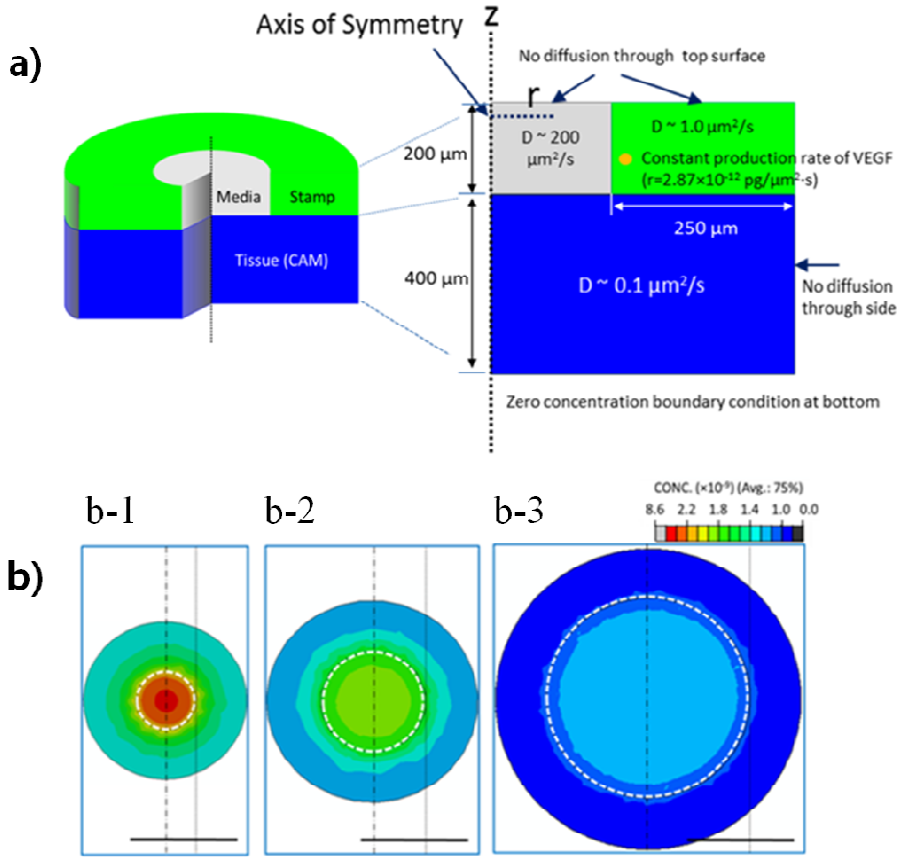

Figure S4. (a) Boundary conditions and the dimensions of the stamp used in the numerical analysis. The top surface and the outer vertical surface of the stamp were assumed to be insulated. The CAM membrane was modeled as being 400 μm thick with a constant concentration of zero at the lower boundary of the system. (b) Numerical analysis of the top view of the VEGF concentration distribution on a plane 20 μm below the hydrogel-CAM interface at Day 7 for three different microchannel diameters (b-1; 300 μm, b-2; 500 μm, and b-3; 1000 μm).

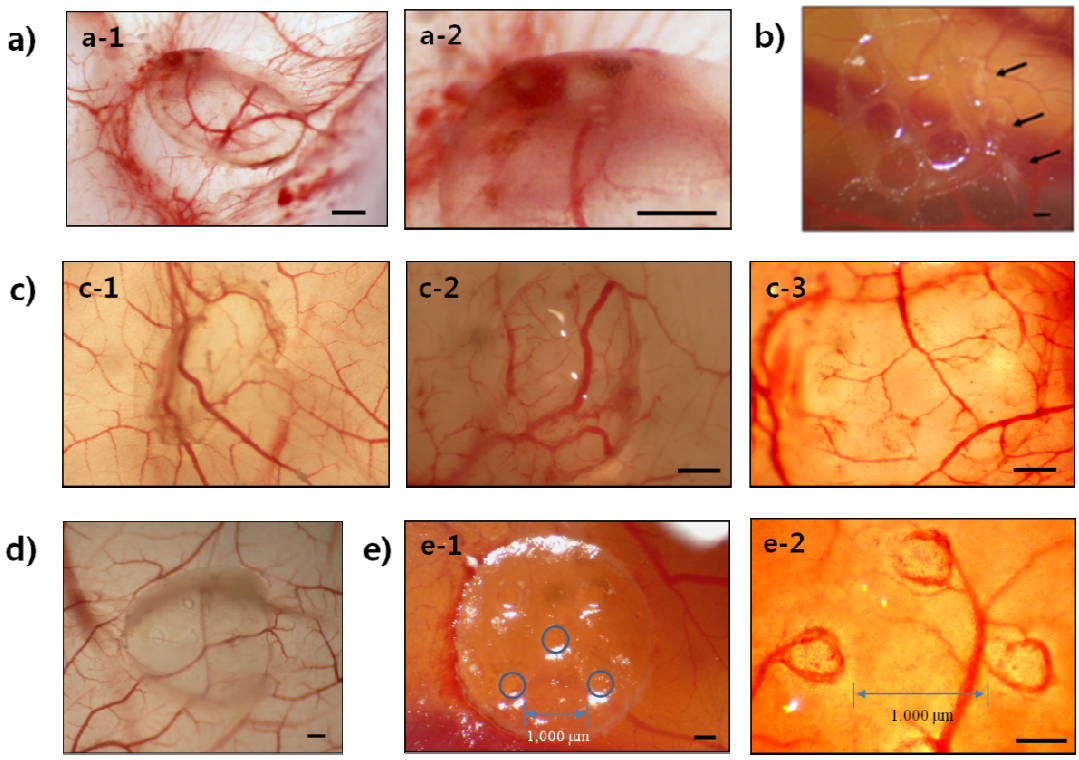

Figure S5. Chick embryo chorioallantoic membrane (CAM) angiogenesis assay. (a) Implantation of the PEGDA hydrogel did not generate capillary patterns, but instead stimulated inflammation. (b) The fractured hydrogel stimulated inflammation, marked by white fibrous tissues around the implant (indicated by an arrow), within two days, while the intact hydrogel only minimally stimulated host inflammation. (c) Patterning of neovessels was not achieved with the cell-free PEGDA-MA hydrogel incubated with PKC activator (c-1), or the hydrogel which contains encapsulated cells at densities of 5×10^4 cells/ml (c-2), and 5×10^5 cells/ml (c-3). (d) Encapsulating only VEGF (30 ng/ml) without fibroblasts did not generate any patterned neovessels. (e) The vascular stamp with a spacing of microchannels of 1.0 mm generated a circular pattern of neovessels with a spacing of 1.0 mm. Scale bars represent 500 μm.

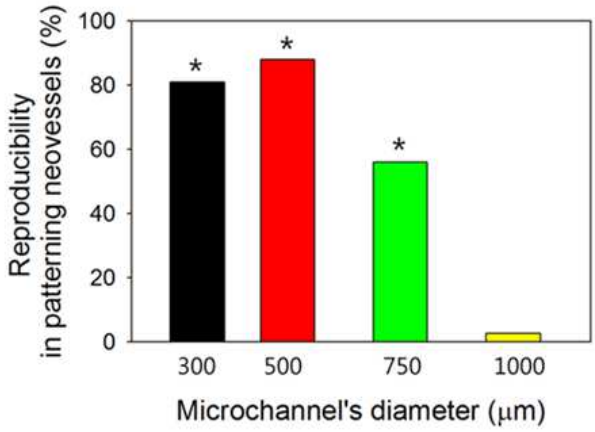

Figure S6. The reproducibility of patterning neovessels, quantified with the number ratio of the patterned neovessels to the microchannels, was related to the diameter of microchannels. The values of the reproducibility in patterning neovessels for microchannel diameters at 300, 500 and 750 μm were statistically different from that attained with microchannel diameter at 1,000 μm (*$p<0.05$). The values of reproducibility represent averaged values from four different samples/CAM assays for each microchannel diameter.

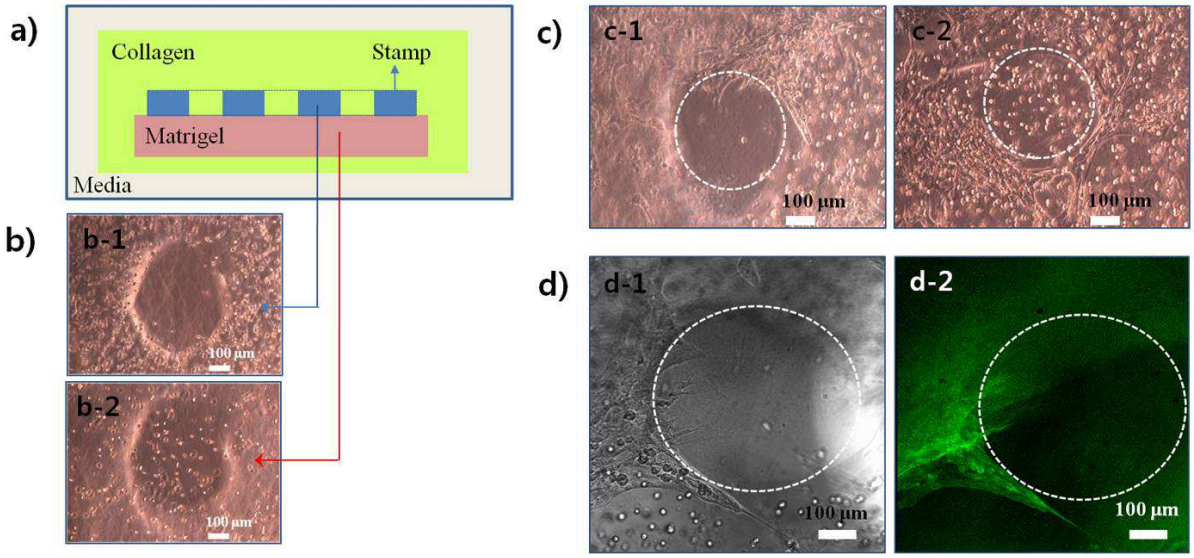

Figure S7. (a) Schematic of the experimental 3D assay to examine directed development of blood vessel-like tubules in 3D hydrogel. Briefly, endothelial cells (C166, ATCC) were cultured on growth factor-reduced (GFR) Matrigel on the MatTek dish. The cell density was kept constant at 5×10^6 cells/ml in each MatTek. The vascular stamp encapsulating fibroblasts was placed on the Matrigel and incubated at 37 °C for 30 minutes to allow it to attach to the Matrigel. Then, the whole gel system was completely embedded in a collagen gel by mixing collagen I solution (3.0 mg/ml, Puramatrix), Dulbecco's Modified Eagle Medium (DMEM, Cellgro), and reconstituting solution (0.26 M of sodium hydrogel carbonate, 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.04 N of sodium hydroxide) at a ratio of 8:4:1. This mixture was subsequently incubated at 37 °C for 4 hours to form a 3D collagen hydrogel. Then, the spatial reorganization of endothelial cells in the 3D Matrigel was examined using the microscope. (b) The first image b-1 shows the fibroblast cells loaded in the vascular stamp, while the next image b-2 shows the endothelial cells loaded in the Matrigel. (c) The vascular stamp encapsulated in the collagen gel stimulated the migration of endothelial cells toward microchannels as shown in the image c-1 and subsequently formed circular patterns of blood vessel-like tubules under the circles of microchannels, as displayed in image c-2. In (c), the white dots represent microchannels in the vascular stamp. (d) Magnified views of image c-2. Image d-1 represents the bright field image of endothelial cells localized around the circumference of the microchannel, and image d-2 represents the endothelial cell lumen stained with fluorescein-phalloidin (green).