Laterally Confined Microfluidic Patterning of Cells for Engineering Spatially Defined Vascularization

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Cell–cell and cell–microenvironment interactions play a key role in directing cellular function. Native tissues and organs are highly organized and possess multiscale architectural features. In the organized structure, cells are embedded within a multicomponent extracellular matrix (ECM).[1] A multiscale vasculature also transfers nutrients and oxygen to the cells.[2] To engineer structured tissues, tremendous efforts have been devoted to engineering materials that can mimic natural ECM and to employ them for generating highly organized constructs.[1,3] Thus, methods including soft-lithography, molding, bioprinting, photo-lithography, and biotextiles have been developed and employed for creating 2D and 3D cellular patterns.[4–10] Although these microfabrication technologies are successful in engineering organized cellular patterns, directing cell growth in cell permissive environments during the long term culture has remained a challenge.

Microfluidic systems have been widely employed for patterning proteins and cells on substrates.[11] Typically, solutions containing proteins or cells are introduced into the polydimethylsiloxane (PDMS) microchannels that are in contact with a substrate to form the patterns; the microchannels are removed after patterning process.[12,13] These systems not only provide a cost-effective and fast method compared to other printing methods, but also they have been shown to be capable of creating multiscale and biomimetic patterns.[12–14] However, similar to other microfabrication techniques, controlling and directing cells to retain the organization over time still remains a challenge as cells migrate from the original defined patterns in a random fashion. Micropatterned surfaces can address such challenge.[15,16] However, these systems are not compatible with a patterning strategy during the seeding process of the cells to precisely define their initial locations.

Here, we have introduced a novel strategy by combining microfluidic patterning and surface microstructuring techniques for creating planar multiscale protein, hydrogel, and cellular patterns, and simultaneously generating microscale topographical features that laterally confine the patterned cells and direct cellular growth in cell permissive hydrogels. We termed our technique laterally confined microfluidic

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DOI: 10.1002/smll.201601391

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patterning (LC-MP) and we have shown that LC-MP can create cellular patterns, and force them to expand vertically to form highly organized 3D architectures. Here, we created multidimensional patterns of human umbilical vein endothelial cells (HUVECs) with a pattern size as small as 50 μm. However, the MP technique can be used to create patterns that are continuous and can support capillarity. Also, the pattern dimensions cannot be smaller than the size of the cells. By applying the concept of capillary-driven systems and eliminating the need for a syringe pump to drive the liquid inside the microchannels, the feasibility of the proposed method was further improved.[17–20] The advantage of the capillary-driven platforms is not only their ease-of-use but also low fluid velocity that dramatically decrease shear stress within the channels (an important factor that affects cell viability).[20,21] In our study, we also investigated the planar organizations and patterns created by LC-MP and studied the possibility of translating them into 3D structures.

MP and LC-MP systems were fabricated using two different procedures (shown in **Figure 1**a). The microfluidic channels were prepared using a soft-lithographic method shown in Figure 1a (the detail of device fabrication is discussed in the Supporting Information). In both methods we made sure that the channels were hydrophilic (to facilitate capillary motion of the liquid inside the channels) and devices were detachable. An example of fabricated PDMS channels (used in this study) with detailed dimensions of the patterns is shown in Figure 1b (i) and (ii). To create LC-MP device, a thin membrane of PDMS was fabricated on the surface of microfluidic device. For this purpose, the surface of the channel was salinized using chemical vapor deposition (CVD) and a thin layer of PDMS was then spin coated (at 6000 rpm for 5 min) on the surface and cured (see Figure 1a). The salinization process significantly reduced the adhesion between the PDMS substrate and the PDMS membrane. The PDMS chip containing microfluidic channels was then plasma treated (to activate the membrane surface and also render the surface of the channels hydrophilic). Thus, upon attachment of the PDMS chip to a plasma activated glass substrate, first the membrane would permanently bond to the substrate and second the formed conduits could easily uptake liquids. To test this hypothesis, the PDMS chip was gently pressed on a membrane on a plasma-activated glass slide. After the patterning process by introduction of aqueous solution (details will be described later), the PDMS chip was gently removed from the surface. The coated membrane was then bonded to the glass and detached from the PDMS chip to create microtopography for directing cellular growth on the glass surface (see Figure 1b (iii)). We also compared the dimensions of the formed architectures on the surface with the intended values (see Figure 1b (iv), where D_C is the width of the PDMS channel and D_S is the width of structures printed on the surface). The thickness of the membrane from the edge of the pattern was measured using a profilometer and shown in Figure 1b (v) (height = 0 corresponds to the elevation of the glass surface). The membrane thickness was less than a micron close to the edge and the thickness increases to approximately 6 μm. The scanning electron microscopy (SEM) image of the membrane confirms that the thickness of the membrane was less than a micron at the edge of PDMS and glass (see Figure 1b (vii)).

The schematic of cell seeding process into the proposed MP system in this study is shown in Figure 1c. In this approach, the LC-MP device (preparation process shown in Figure 1a) and the glass substrate were both plasma treated and bonded together. This will make the surface of the channels hydrophilic, which facilitates the use of capillary microfluidics to drive the liquid inside the channels. A solution of 10 (μg mL^{-1}) of fibronectin was then introduced to the inlet port and incubated for 1 h. After that, the solution was removed using a sterile gauze and a suspension of HUVECs (10^7 cells mL^{-1}) in endothelial cell growth medium (EGM-2) was introduced to the inlet port. After 1 h (when the cells attached to the surface), the microfluidic chip was detached from the surface leaving a PDMS membrane on the surface that laterally confines the patterned cells (see Figures 1b (iii), c (vii) and c (viii)). The patterns were then submerged in EGM-2 media and cultured for 1 d. Finally, a hydrogel layer was casted on the patterns and the constructs were continued for culture.

To demonstrate the operation of the MP platform and ensure that cells can be transported and uniformly patterned within the channels, an aqueous solution containing green florescent particles with sizes comparable to eukaryotic cells was used to create a pattern. It can be seen that the particles are robustly transported and uniformly distributed through the entire length of the channels (shown in Figure S1 (i), Movie 1, Supporting Information). To show the possibility of patterning a second layer of particles on the first layer, a solution containing yellow particles was introduced to the channel (after sedimentation of the green particles) and patterned on the first layer (Figure S1 (ii), Supporting Information). The feasibility of the MP platform for engineering high-resolution patterns from proteins, cells, and hydrogels was also assessed. We created fibronectin and green fluorescent patterns on glass substrates (Figure S1 (iii), Supporting Information). Similarly, gelatin methacryloyl (GelMA) precursor was introduced to the MP platform and was crosslinked by UV illumination (Figure S1 (iv), Supporting Information), then the PDMS microchannel was simply removed leaving behind a hydrogel construct as the hydrogel has higher adhesion to hydrophilic surface of glass than hydrophobic surface of the PDMS channels. It is also shown that MP was capable of creating hydrogel patterns on a hydrogel substrate (Figure S1 (v), Supporting Information). In this case, 3D patterns of photocrosslinkable polyethylene glycol diacrylate (PEGDA) hydrogel on an already crosslinked PEGDA substrate. Likewise, GelMA hydrogel was patterned on a GelMA substrate (SEM images of the freeze-dried hydrogel sample are shown in Figure S1 (vii) and (viii), Supporting Information). The generated seamless patterns confirm the robustness of the technology in engineering planar architectures from particles, proteins and hydrogels.

We also assessed the possibility of creating cellular patterns using the MP-platform. To test the feasibility of the platform for engineering high-resolution vascular-like patterns, fibronectin was loaded into the inlet port of the microfluidic chip covered by a glass substrate. After incubation for 30–60 min, the solution was removed using a sterile fabric

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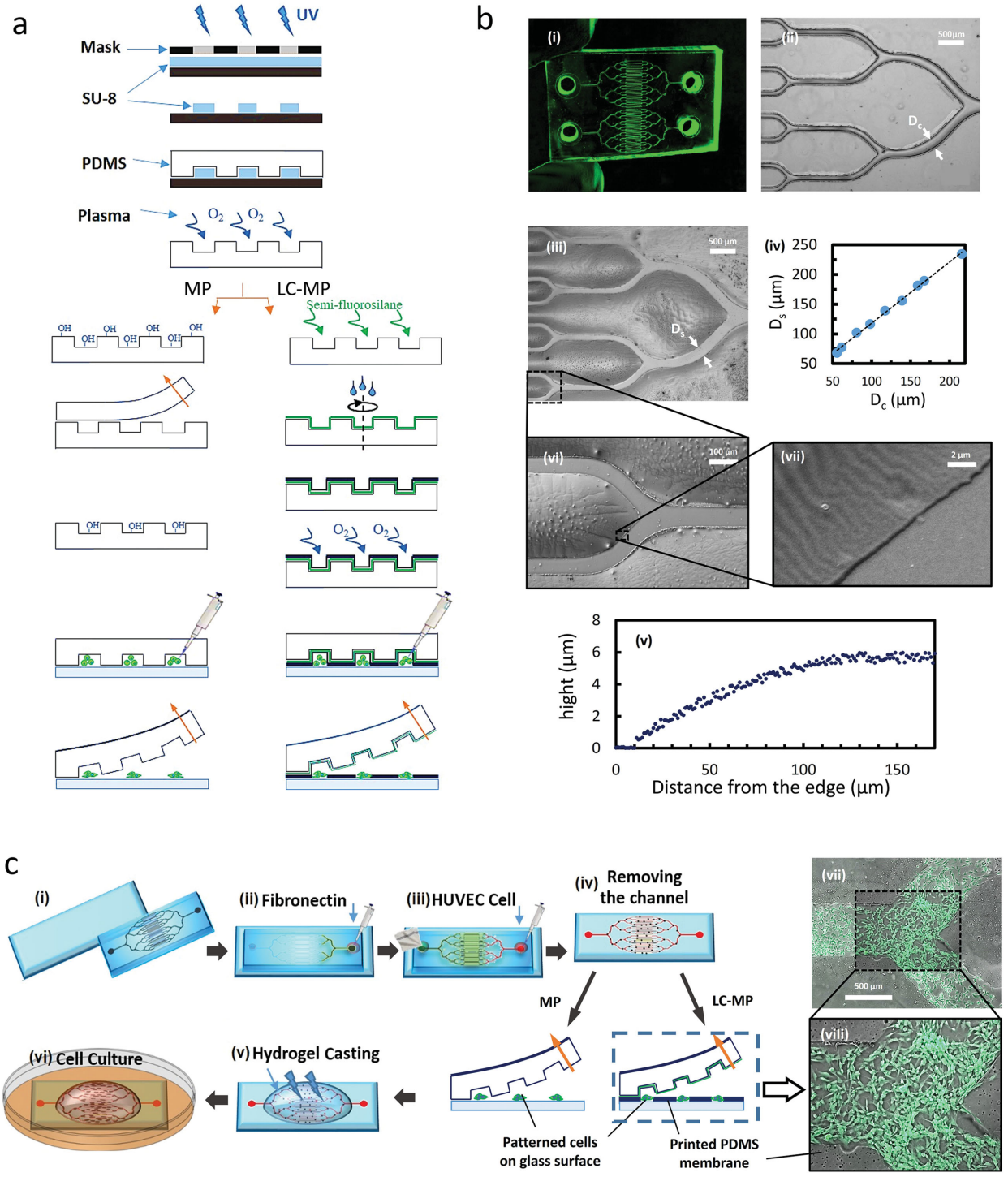

Figure 1. Demonstration of laterally confined microfluidic patterning method used for cell and protein patterning, and hydrogels. a) Schematic of the fabrication procedure followed to make MP and LC-MP devices. b) (i) Microfluidic PDMS channel device with (ii) detailed dimension of the channels (D_C is defined as the width of the PDMS channels). (iii) PDMS membrane printed on glass surface (D_S is defined as the width of the printed microstructure created on the surface). (iv) The width of the printed membrane pattern on the surface (D_S) versus the width of its corresponding PDMS channel (D_C) is depicted. (v) The profile of membrane was measured with respect to the edge of membrane using a profilometer (height = 0 is the glass surface). (vi) Magnified image of a printed junction on a glass substrate. (vii) SEM image of the patterned PDMS membrane on a glass, showing the thickness of the PDMS membrane at the edge is less than a micron. c) Initially, (i) the glass substrate and the microfluidics channels are oxygen plasma treated. (ii) Microfluidic PDMS channels are assembled on the glass substrate and fibronectin solution is loaded into the inlet port. (iii) After 1 h incubation, the solution is collected from the outlet and meanwhile the solution containing HUVECs is loaded in the inlet (forced capillary). (iv) After 1 h incubation, the PDMS channel is removed and cultured for 1 d before casting the hydrogel. (v) Hydrogel is casted on the sample. (vi) The glass substrate is then immersed in the cell media and cultured. (vii) Patterned HUVECs on the glass surface (using LC-MP method) with PDMS membrane confining the cells. (viii) Magnified image of the patterned cells and the PDMS membrane.

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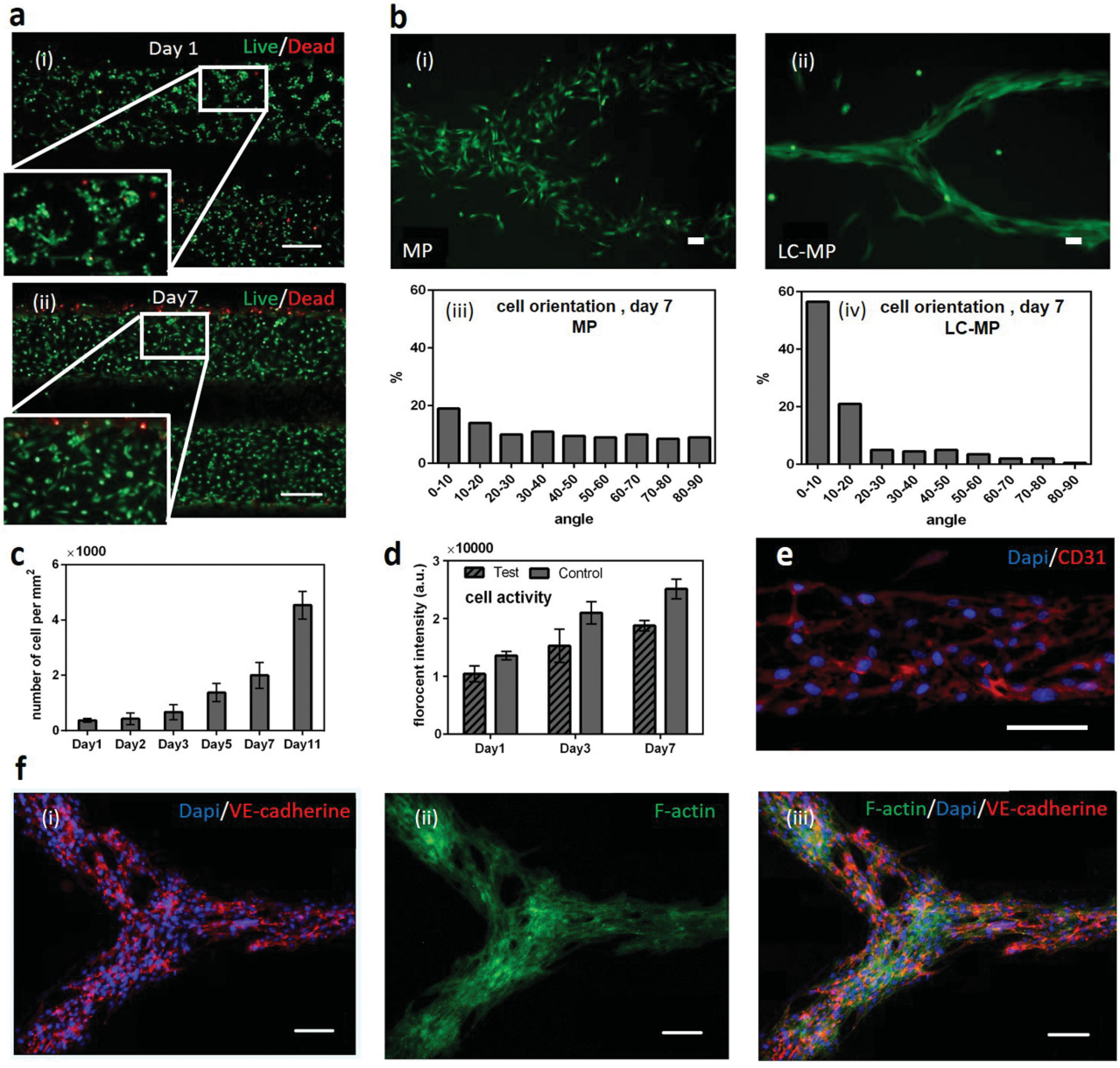

Figure 2. Biological study of the patterned network of HUVECs using LC-MP technique for samples with casted 6.5% (w/v) GelMA hydrogel. a) LIVE/DEAD assay performed for (i) day 1 and (ii) day 7. b) Comparing the patterning quality achieved after 7 d of culture for (i, iii) MP and (ii, iv) LC-MP methods. The orientation of the cells was quantified for (iii) MP and (iv) LC-MP methods. c) Density of the cells in the pattern. d) Cellular activity measured with Prestoblue assay and compared to control. e) CD31 protein expression after 7 d of culture shown in red color and the cell nucleus shown in blue. f) VE-Cadherin expression (shown in red color) from HUVECs at day 11 of the culture. (i) Red (VE-Cadherin) and blue (cell nucleus) channels. (ii) Green channel showing actin filaments. (iv) Merge of red, blue, and green channels. The scale bars are showing 100 µm.

and a solution of HUVECs was introduced to the inlet port. The structures were incubated for 1–2 h and then the glass was detached from the PDMS chip. We investigated the viability of cells patterned using MP-platform after 1 and 7 days of culture using a Live/Dead Assay Kit (Invitrogen) (**Figure 2**a). Approximately 95% viability was observed at days 1 and 7, which confirms the biocompatibility of the method (Figure 2a). In addition to creating cellular patterns, we investigated the possibility of maintaining and directing cellular behavior and growth using the MP-platform (Figure 2b). We compared the patterning quality and behavior of cells within the patterns engineered using MP (Figure 2b (i)) and LC-MP (Figure 2b (ii)). Although both methods were able to initially create fine patterns, significant cell migration was observed in the samples patterned using MP technique after 7 days and no oriented structures were observed (Figure 2b (iii)). In contrast, in the samples that were patterned using LC-MP, cellular organization was preserved and cell migrations were mostly through sprouting process essential for formation of micro-capillaries (Figure 2b (ii)). It was observed that LC-MP patterning significantly enhanced cellular alignment. More than 55% and 75% of cells were aligned with <10° and <20° deviation from the direction of the micropatterns on day 7 (Figure 2b (iv)). As a

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result, the formation of highly oriented and tightly connected cellular structures similar to the initial patterns was observed.

The average cell density in the patterns was calculated for 1, 3, 5, 7, and 11 d of culture (Figure 2c). The density of the cells increased from ~370 cell mm^{-2} at day 1 to ~670 and ~4500 cell mm^{-2} at days 3 and 11, respectively. The metabolic activity of cells was determined after 1, 3, and 7 d of culture using Prestoblue assay (Figure 2d). Cells showed a similar growth rate and the metabolic activity to the control after 1, 3, and 7 d, respectively.

Immunostaining was also carried out to investigate the expression of endothelial cell markers. CD31 and 4′,6-diamidino-2-phenylindole (DAPI) were used to stain HUVECs and nuclear counter stain in the patterned network, respectively (Figure 2e). The formation of tight junctions by HUVECs in the pattern was examined at day 11 of the culture by observing the expression of vascular endothelial Cadherin (VE-Cadherin) protein (Figure 2f). The expression of tight junctions is an indication of proper alignment and cell–cell interaction, which is an important parameter in the formation of functional vasculature (Figure 2f).

The engineered multiscale patterns facilitate characterization of geometrical parameters impact on cellular morphology and behavior. Thus, we created endothelial cell cultures as described before using the LC-MP and were covered with GelMA hydrogel. To study cellular morphology, the nucleus and cytoskeletal actin filaments alignment of the multiscale HUVECs patterns covered with GelMA hydrogel were stained using DAPI and phalloidin after 7 and 11 d of culture (**Figure 3**). Significant increase in cell proliferation and density was observed between days 7 and 11, whereas little cell migration from the original-pattern was observed up to day 7 (Figure 3a). However, significant number of sprouting branches were observed at day 11 (Figure 3c), which were able to create connection between pattern lines. Although sprouting was observed in all pattern sizes, patterns with width smaller than 80 μm showed the maximum cell alignment and higher sprouting numbers after day 7 (Figure 3a). In GelMA casted samples, the size of sprouts and cell density in each branch increased gradually after day 7.

As reported by others, smaller dimensions led to faster cellular alignment. For example, cells were aligned in the patterns with less than 80 μm width (Figure 3b). Random orientation in different directions was observed in wider patterns with width above 150 μm at day 7 (Figure 3c). However, cell orientation (especially cytoskeleton orientation) was improved until day 11 in wider patterns as well (Figure 3d). This can be explained due to the higher density of cells at day 11 (~4500 cell mm^{-2}), which was 2.25 times higher than the values at day 7 (Figure 2c).

To investigate the possibility of translating the 2D patterns into 3D construct, we covered the patterns created using LC-MP with a thin layer of hydrogels including GelMA, Matrigel, and Collagen type I. **Figure 4**a shows that in the cellular patterns covered with Matrigel, cell migration toward the gel was significant, which was different from cellular behavior in the samples covered by GelMA (Figure 3). Cells formed thin branches without following the initial 2D pattern. HUVECs in Matrigel exhibited more cell elongation similar to Epithelial-shape (Figure 4a).

Less cell migration was observed in the pattered cultures covered with collagen and the initial pattern organization was preserved (Figure 4b). The cells in collagen had spindle shape and remained fully oriented with thick and regular sprouting from patterns. In comparison with GelMA casted samples (Figure 3a), the HUVECs formed more homogenous structures with rare discontinuity in the network in the collagen casted samples (Figure 4b). To quantitatively study the cell behavior, density (Figure 3c) and elongation (Figure 3d) of HUVECs in GelMA, Matrigel and collagen samples were calculated at day 7 of the culture. The lowest cell density (~1670 cells mm^{-2}) and the highest cellular elongation (length/width = 9.5) were observed for Matrigel. Collagen samples, on the other hand, showed the highest cell density (~3700 cells mm^{-2}) and the lowest cellular elongation (length/width = 3). Confocal microscopy images showed that HUVECs patterned using LC-MP initially formed cord-like structures (two layers of cell sheets on each other). Subsequently, the cells created hollow lumen-like tubes (see Figure 4f). The lumen-like structure was mostly observed in narrow patterns and the sprouting branches (Figure 4d). The main focus of this study has been on the development of methods creating patterns that are preserved as cells proliferate. We imaged the cells after 7 d of culture and noticed that the pattern was preserved and cells have migrated into the gel following the initial pattern. Also the sprouts shown in Figure 4f confirm the organized migration of the cells in 3D.

In conclusion, we have successfully developed a robust method for engineering hierarchical and biomimetic patterns from proteins, cells, and hydrogels. In this method, a capillary driven microfluidic approach is used to drive different solutions (e.g., hydrogel, protein and solutions containing particles and cells) into the patterned channels. To be able to direct cellular growth and preserve the desired patterns, we combined the method with molding techniques and developed a LC-MP approach. Our method enables creating patterns and preserving them in a single step. In fact, the microfabricated pattern can be used multiple times, while in lithography-based methods each sample should be individually microfabricated and still preservation of cellular patterns over time is not trivial. The proposed method enables patterning and preservation in a single step. We confirmed the biocompatibility of the process and also investigated the possibility of translating the planar patterns into 3D hydrogels. It was observed that covering the patterned with a cell permissive hydrogel can lead to formation of 3D architectures. The hydrogel properties play a key role on the cellular behavior and by utilizing the proper environment the planar patterns can be translated into 3D environment.

Experimental Section

Materials: Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise. GelMA was prepared using the method and conditions described in the previous work.[13] Briefly, type-A porcine skin gelatin was

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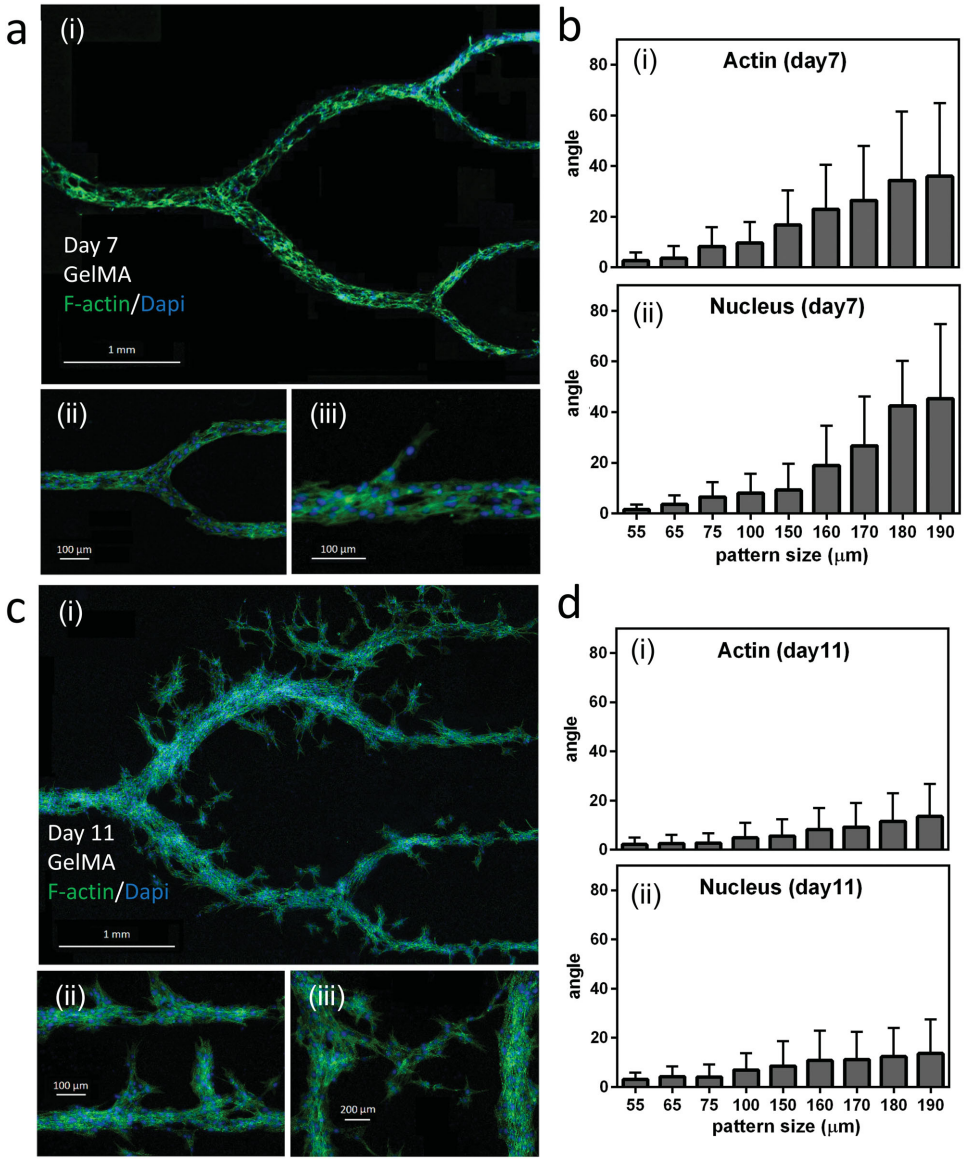

Figure 3. Actin/Dapi staining of HUVEC network using LC-MP technique for samples with casted 6.5% (w/v) GelMA hydrogel. a) Staining of HUVEC culture for F-actin in the network after 7 d of culture (i) Continuous network of HUVECs. (ii) Tuning fork-shape junction splitting a 65 µm line into two 55 µm lines. (iii) Sprouting in 65 µm line. b) Orientation of cell (i) cytoskeleton and (ii) nucleus calculated after 7 d of culture. c) Staining of HUVECs in the network after 11 d of culture showing significant number sprouting. (i) Continuous network of HUVEC culture for F-actin showing significant number of sprouts branches in all pattern sizes. (ii) Thick sprouting branched grew toward other lines in the network. (iii) Interconnection between two lines through sprouting branches. d) Orientation of (i) actin and (ii) nucleus measured after 11 d of culture.

dissolved in PBS at 60 °C. Methacrylic anhydride (MA) was added drop wise to the gelatin solution under continuous stirring. The solution was dialyzed against deionized water using 12–14 kDa cut-off dialysis tubes at 50 °C for 7 d to remove unreacted MA. The solution was then freeze-dried and stored at room temperature for further use. GelMA pre-polymer solution was mixed with 0.25% (w/v) photoinitiator (PI) and exposed to UV light (850 mW) for 30 s to crosslink. Cell culture reagents and assays were purchased from Invitrogen (Carlsbad, CA, USA). Acrylic florescent pigments (Americana Neons fluorescent acrylic paint) were purchased from DecoArt, Inc. (Stanford, KY, USA) and used as the florescent particles in the experiments.

Patterning of Proteins, Cells, and Hydrogels: After preparation of the PDMS microchannels, the PDMS and the glass substrate were plasma treated and bonded together. Initially, 300 µL of 10 (µg mL^{-1}) fibronectin solution was dispensed into the inlet port. The solution flowed inside the channel network due to the capillary forces and reached to the outlet port of the device. The device was then placed in an incubator for 30–60 min to increase the physical adsorption of fibronectin to the glass surface. To culture cells, HUVECs suspended in culture medium were introduced into the channels at the concentration of 10 M mL^{-1}. The sample in the outlet port was collected by a sterile medical gaze causing the flow of the liquids toward the outlet port (force capillary motion). The device was then observed under a microscope to make sure of the quality of the cell patterning process. The system was then placed in an incubator for 60 min to allow cell adhesion to the fibronectin coated surface. In the next step, the PDMS chip was removed from

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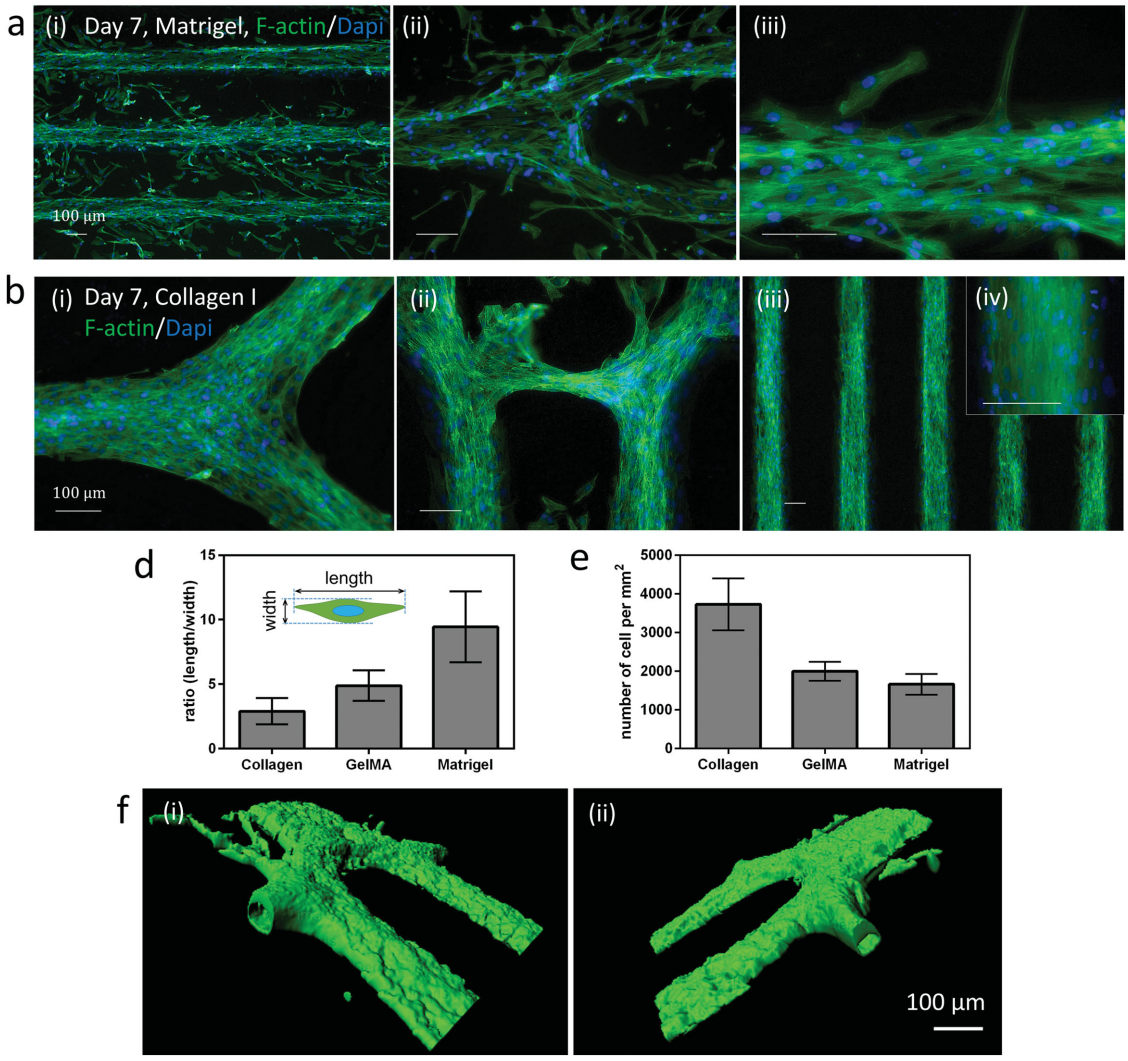

Figure 4. Study of different hydrogel on cellular behavior. a) Actin/Dapi staining of HUVECs in the network for Matrigel at day 7, (i) significant cell migration was observed from the patterns. (ii) Tuning fork-shape junction in Matrigel connecting a 65 µm line to two 55 µm lines (the numbers are for the channel size used to create the patterns), (iii) magnified image of a 65 µm line of HUVECs (initial size) showing a cell extending itself toward the other line. b) Actin/Dapi staining of HUVEC network for collagen type I at day 7. (i) A Y-shape junction with high cell density and very uniform cell morphology. (ii) Interconnection between two lines through a thick sprouting branch. (iii) Lines of patterned HUVECs in Collagen with homogenous morphology. (iv) Magnified image showing one of the patterned lines. d) Cell elongation in different hydrogels. e) Cell density in samples with different casted hydrogels. f) Confocal image of a junction in collagen hydrogel that has two sprouting branches at (i) left and (ii) right sides. Both sprouting branches showed a hollow structure.

the glass slide leaving pattern of cells on the glass surface. Finally, 1–2 mL of EMG-2 media was added to top of the glass slide. The glass slide was then placed in the incubator for culturing.

For patterning of hydrogel constructs, the same approach as described above was followed to activate the glass slide and PDMS chip; thereafter, GelMA precursor solution was introduced into the network and once the channels were filled, the system was exposed to UV for 60 s. The PDMS chip was then removed leaving behind the hydrogel construct.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

H.R.N. and Z.G.M. contributed equally to this work. The authors declare no conflict of interests in this work. This work was supported by National Science Foundation (EFRI-1240443), the Office of Naval Research Young Investigator award, ONR PECASE Award, and the National Institutes of Health (HL092836, DE019024, EB012597, AR057837, DE021468, HL099073, EB008392). The project was also funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, under Grant No. 18-130-1434-HiCi. Y.S.Z. acknowledges the National Cancer Institute of the National Institutes of Health Pathway to Independence Award (1K99CA201603-01A1). H.R.N. acknowledges The Constance Livingstone-Friedman and Sydney Friedman Foundation Scholarships in Health Sciences.

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Received: April 22, 2016
Revised: June 8, 2016
Published online: August 11, 2016

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