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Rapid homogeneous endothelialization of high aspect ratio microvascular networks

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Abstract Microvascularization of an engineered tissue construct is necessary to ensure the nourishment and viability of the hosted cells. Microvascular constructs can be created by seeding the luminal surfaces of microfluidic channel arrays with endothelial cells. However, in a conventional flow-based system, the uniformity of endothelialization of such an engineered microvascular network is constrained by mass transfer of the cells through high length-to-diameter (L/D) aspect ratio microchannels. Moreover, given the inherent limitations of the initial seeding process to generate a uniform cell coating, the large surface-area-to-volume ratio of microfluidic systems demands long culture periods for the formation of confluent cellular microconduits. In this report, we describe the design of polydimethylsiloxane (PDMS) and poly (glycerol sebacate) (PGS) microvascular constructs with reentrant microchannels that facilitates rapid, spatially homogeneous endothelial cell seeding of a high L/D (2 cm/35 μm; > 550:1) aspect ratio microchannels. MEMS technology was employed for the fabrication of a monolithic, elastomeric, reentrant microvascular construct. Isotropic etching and PDMS micromolding yielded a near-cylindrical microvascular channel array. A 'stretch – seed – seal' operation was implemented for uniform incorporation of endothelial cells along the entire microvascular area of the construct yielding endothelialized microvascular networks in less than 24 h. The feasibility of this endothelialization strategy and the uniformity of cellularization were established using confocal microscope imaging.

Keywords Engineered microvascular construct · Endothelial cell seeding · MEMS · Micromolding

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1 Introduction

A substantial effort has been directed towards the development of vascularized tissues to ensure the nourishment and viability of engineered 'living' tissues. Although various approaches have been investigated for the creation of individual vascular conduits with diameters that exceed 1 mm, recent applications of MEMS-based techniques has facilitated the development of microvascular networks. Micromachining strategies have afforded microchannel fluidic devices with complex shapes necessary to recapitulate a cell-seeded 'living' microvasculature (Borenstein et al. 2002; Christina et al. 2005; Deniz and Chang 2000; King et al. 2004; Rosano et al. 2009; Shin et al. 2006; Wang et al. 2005).

The lumen of a native blood vessel is lined with a monolayer of non-thrombogenic endothelial cells. In the absence of an endothelial cell layer, small-diameter vascular conduits exhibit a high incidence of thrombosis with poor long-term patency. Reports suggest that endothelialized vascular grafts display a reduced thrombotic response as compared to non-seeded grafts (Pasic 1996). Hence, several static and dynamic endothelial cell seeding strategies have been explored for the construction of a confluent endothelium on the luminal surfaces of vascular grafts (Burg et al. 2000; Hsu et al. 2005).

Endothelial cell seeding of microvascular networks relies on perfusion-based approaches and is a function of length-to-diameter (L/D) aspect ratio of the microchannels and cell diameter-to-channel diameter ratio (*d*/D) (Kim et al. 2009; Wyss et al. 2006). High L/D and *d*/D ratios may cause non-uniform cell distribution along the length of the microchannels, cell agglomeration, and hence microvascular channel plugging (Yap et al. 2006). The clogging phenomenon is observed to be most pronounced for *d*/D>1/3 limiting this approach to microchannel dimensions greater than three times the cell diameter (D>60 μm) (Abdelgawad et al. 2008; Hallow et al. 2008). Approaches explored to enhance the uniformity and efficiency of cell seeding of small-bore (<6 mm) vascular grafts have included the use of dynamic perfusion-based systems, vacuum, and radial magnetic fields applied to cells tagged with magnetic micro/nanoparticles (He et al. 2009; Ito et al. 2005). Although these methods have demonstrated success for conduits with diameters in the range of 4 to 6 mm, they may not be suitable for the seeding of microvascular conduits. Even highly deformable cells can result in flow obstruction or plugging of microchannels and the use of magnetic particles can potentially induce cell toxicity. Likewise, the generation of radially uniform fluidic or magnetic force fields throughout a two-dimensional microvascular network may be somewhat challenging. Further, such approaches subject cells to substantial mechanical stress that could induce cell injury or death.

Microvascular networks, characterized by very high surface area-to-volume ratios, are cellularized with typical seeding densities of $1–5\times10^7$ cells mL^{-1} (Borenstein et al. 2002; Khademhosseini et al. 2004; Wang et al. 2005). Potential channel plugging precludes the use of a higher seeding density (Khademhosseini et al. 2004). Consequently, constructs require days to weeks to reach confluence throughout the entire microvascular area (Borenstein et al. 2002; Wang et al. 2005).

Microvascular channels fabricated using soft lithography generally exhibit rectangular cross sections. This physiologically atypical geometry inhibits conformal cellular proliferation and leads to non-uniform flow profile (Camp et al. 2008). Other efforts to fabricate microvascular networks with circular cross-sections have involved alignment bonding of complimentary hemicylindrical microchannels (Borenstein et al. 2010). However, this approach may not be suitable for construction of densely packed microchannels with diameters of ~10 μm, characteristic of native capillary microvessels.

The current research presents a deformable reentrant microvascular construct supporting a 'stretch-seed-seal' strategy for enhanced spatial homogeneity of endothelialization of a network of sub-50 μm microchannels, as illustrated in Fig. 1 (Naik et al. 2011). The width of the cleft on the sealing wall of the reentrant microchannels was designed to be smaller than the cell diameter such that cells could be introduced into the microchannels when the cleft was in the open state (under tension), while preventing cell loss when the cleft closed in the relaxed state. To enable endothelial cell seeding, the device was stretched to widen the clefts followed by relaxation of the elastomeric constructs. Seeded cells were then allowed to adhere, proliferate, and conformally line the microvascular channels. It was postulated that under conditions of high seeding density, this approach would allow rapid and uniform cell deposition along the entire length of multiple microchannels.

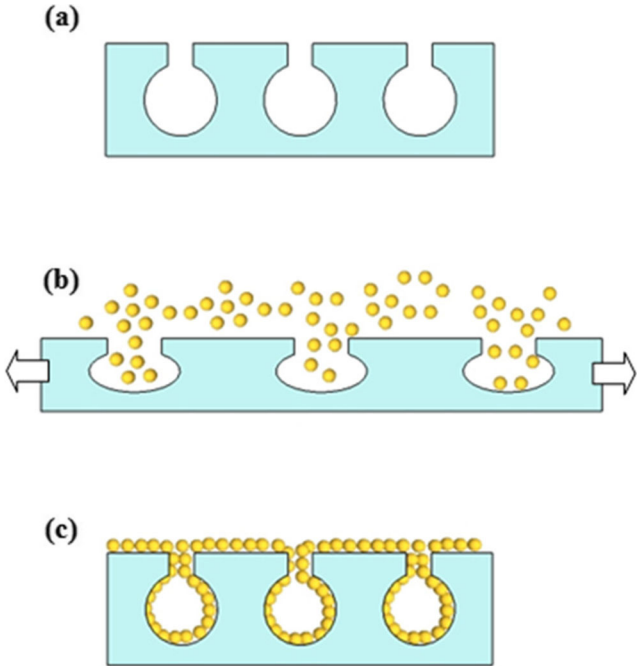

Fig. 1 A schematic illustration of the 'stretch - seed - seal' strategy for endothelialization of a reentrant microvascular construct. **a** Microvascular construct in the relaxed state. **b** Stretch and seed cells in the open state for concurrent deposition through the entire length of the microchannels. **c** Release the construct back to the relaxed state and culture cells to form a spatially uniform endothelial cell layer

2 Materials and methods

2.1 Materials

Polydimethylsiloxane (PDMS) elastomer, Sylgard 184, was obtained from Dow Corning. Poly(glycerol sebacate) (PGS) was kindly provided by Prof. Yadong Wang from the University of Pittsburgh. Human umbilical vein endothelial cells (HUVECs) and endothelial cell basal medium (EBM) supplemented with endothelial growth medium (EGM) SingleQuots were purchased from Lonza. Green fluorescence, CellTrace CFSE, was purchased from Invitrogen. Fibronectin was purchased from Sigma-Aldrich.

2.2 Microfabrication of PDMS and PGS reentrant microvascular constructs

MEMS processing technology was utilized for the development of a monolithic network of near-cylindrical, reentrant microvascular channels, as shown in Fig. 2. The spatially controllable and dimensionally scalable microvascular patterns were defined on a silicon wafer using photolithography. Positive photoresist AZ4620 (Hoechst Celanese, Somerville, NJ) was spin-coated and patterned on a (100) silicon wafer. Photoresist thicknesses of 15 and 8 μm were used for microvascular constructs with cleft widths of 12 and 6 μm, respectively. A combination of anisotropic inductively coupled plasma (ICP) etching and isotropic xenon difluoride (XeF2) etching steps were performed to create a silicon-photoresist mold with near-unity depth-to-width (D/W) aspect ratio microchannels (Fig. 3). The microvascular patterns were first etched into the silicon wafer anisotropically using ICP (Bosch process, SF_6/C_4F_8) to a depth i. The microchannels were subsequently etched using XeF_2 to a further depth, x_v, such that

$$x_v = i - c_0$$

where, x_v is the vertical etch depth using xenon difluoride etching, x_l is the lateral etch width using xenon difluoride etching, i is the etch depth using ICP etching, and c_0 is the cleft width. The vertical and lateral etch rates x_v and x_l were assumed to be roughly equal in this model.

A 1 μm thick film of parylene was deposited on the resultant silicon-photoresist mold to facilitate subsequent PDMS release. The patterns from silicon-photoresist mold were transferred to a PDMS negative master mold. PDMS was cast on the silicon-photoresist mold, degassed in vacuum, and cured at 60 °C for 10 h. PDMS curing agent to pre-polymer base ratios of 1:10 and 1:5 were used for microvascular constructs with cleft widths of 12 and 6 μm, respectively. These ratios have been previously shown to mantain endothelial cell markers, viability, and proliferation (Esch et al. 2011; Lee et al. 2004). The PDMS negative master mold was gently demolded from the silicon-photoresist mold. Demolding of the master mold was accomplished by fracture of the overhanging photoresist walls. The reusable master mold surface was passivated with an anti-stiction film by exposing it to trichloro(1H,1H,2H,2H-perfluorooctyl) silane vapor (Sigma Aldrich) to prevent PDMS adherence. For

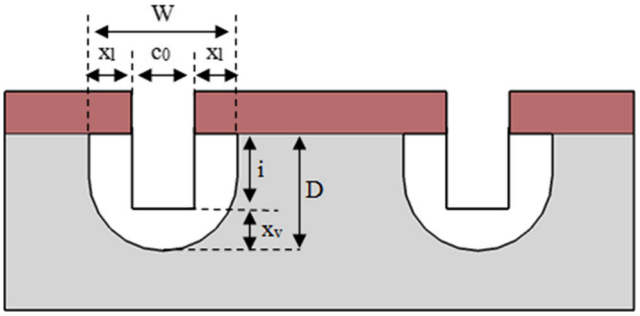

Fig. 3 Inductively coupled plasma and xenon difluoride etching for the fabrication of a near-unity aspect ratio microchannel silicon-photoresist template

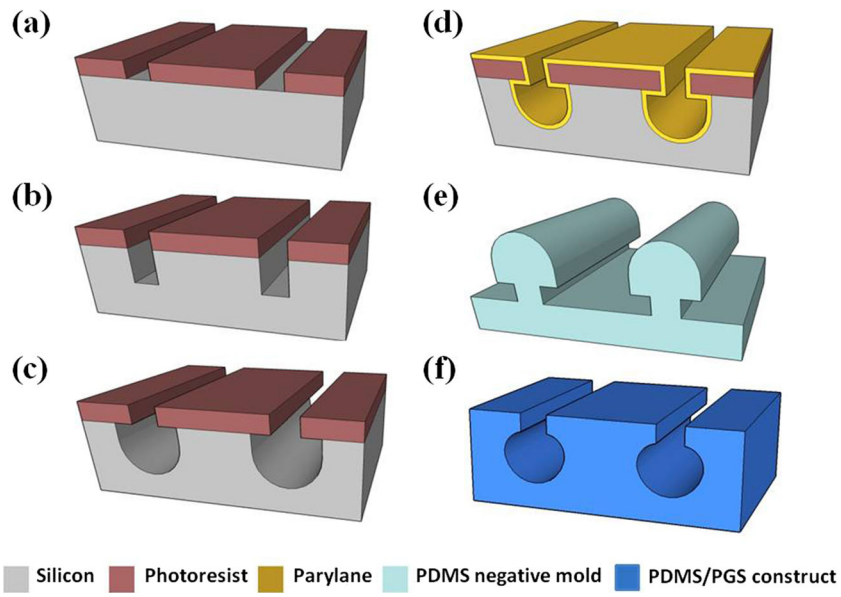

Fig. 2 Fabrication process for reentrant PDMS and PGS microvascular constructs. **a** Photoresist is spin-coated and patterned on a silicon wafer. **b** Microchannels are anisotropically etched into the silicon wafer using ICP. **c** The silicon channels are etched isotropically using XeF_2 etching to create a 1:1 aspect ratio microchannels. **d** A 1-μm thick layer of parylene is deposited on the silicon- photoresist template. **e** PDMS is poured onto the template and cured at 60 °C. The reusable PDMS negative mold is released from the silicon-photoresist mold. **f** PDMS micromolding is used to fabricate PDMS and PGS reentrant microvascular constructs

the fabrication of PDMS reentrant microvascular constructs, PDMS (curing agent to pre-polymer base ratio of 1:10) was cast on the master mold, degassed in vacuum, cured at 60 °C for 10 h, and released. PGS reentrant microvascular constructs was fabcricated with some modifications from a previoulsy published protocol (Christina et al. 2005). Briefly, 90 % sucrose solution (0.2-μm pore size filtered) was spincoated into the PDMS master mold (1000 rpm for 30 s) and air-dried to create a thin sacrificial layer. Then, 0.1 g of prepolymer PGS was melted and poured over the PDMS master mold. The prepolymer was cured at 120 °C for 48 h. The PDMS master mold was then soaked in deionized water for 24 h to facilitate the released of the PGS reentrant microvascular constructs. Any residual oligomers or unreacted starting materials were removed by soaking the PGS reentrant microvascular constructs in a gradient of ethanol (90–10 %) for 24 h.

2.3 Reentrant construct preparation for cellularization

In preparation for cell seeding, the PDMS and PGS reentrant microvascular constructs were hydrophilized with a handheld corona discharge tool (Electro-technic product, Il) for 60 s (Haubert et al. 2006), sterilized in ethanol for 30 min, and rinsed twice with sterile molecular grade water. The construct was functionalized with fibronectin to promote cellular adhesion by incubating in 50 μg mL^{-1} fibronectin at 37 °C for 2 h. It was then washed with 5 mL of phosphate buffer saline (PBS) and placed in endothelial cell basal medium at 37 °C for 30 min prior to cell seeding.

2.4 Endothelial cell seeding

HUVECs were thawed and cultured in a 75 cm^2 polystyrene flask with endothelial cell basal medium supplemented with endothelial growth medium SingleQuots. Adherent HUVECs were pre-labeled with green fluorescence (CellTrace CFSE in HBSS, 0.7 uM) at 37 °C for 15 min. Pre-labeled HUVECs were incubated in full serum media at 37 °C for 1 h, trypsinized, neutralized with media, spun down to a pellet, and resuspended in full serum media at 1×10^6 cells mL^{-1}.

An aluminum tensile loading apparatus was used to load the reentrant microvascular construct in tension to widen the clefts, as shown in Fig. 4. The apparatus consisted of two clasps to secure the ends of the construct and a tensile loading screw for the application of a tensile strain. The construct was stretched open using applied strains of 50–100 % depending on the channel and cleft widths. Upon tensile load application, an acrylic container was mounted over the microvascular network to enclose cells within a defined seeding area. HUVECs in full serum media (100–150 μL/construct, 1×10^6 cells mL^{-1}) were introduced onto the construct in the open state and the tensile loading apparatus was placed in an incubator at 37 °C and 5 % CO_2 for 4 h. The construct was rinsed with 10 mL of HBSS to remove non-adherant cells and tension released. Constructs with 12 μm cleft widths were re-seeded at 250,000 cells cm^{-2} to promote rapid cleft sealing. Constructs with 6 μm cleft widths did not require an additional seeding step. A total of 5 mL of EBM was introduced and cells cultured at 37 °C for up to 24 h.

2.5 Microscopic analysis

The silicon-photoresist molds, PDMS master molds, and PDMS and PGS reentrant microvascular constructs were imaged using a scanning electron microscope (SEM, JEOL 5910) and an inverted microscope (Nikon Instruments). Fluorescence images of the endothelialized constructs at prescribed time intervals were acquired using confocal microscopy (Ex/Em: 492/517). Three-dimensional reconstructions were performed using ImageJ and Imaris software.

3 Results and discussion

3.1 Fabrication

The fabricated molds and reentrant constructs are shown in Fig. 5. The developed fabrication approach yielded

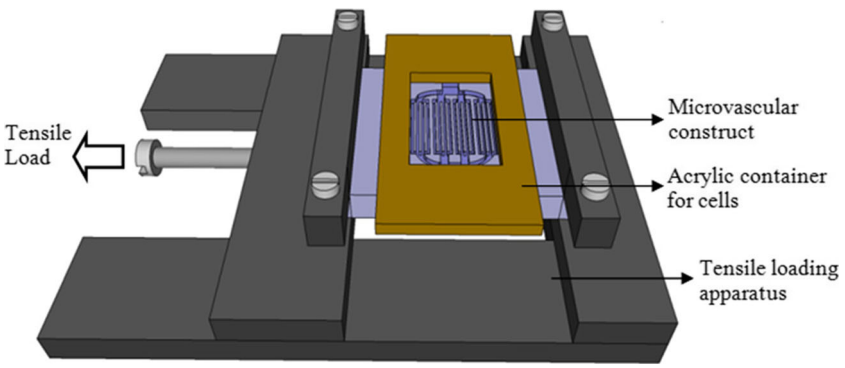

Fig. 4 A tensile loading apparatus for widening the microchannel clefts prior to endothelial cell seeding. The reentrant microvascular construct is secured in the apparatus using clasps. A strain of 50-100 % is applied using a tensile loading screw

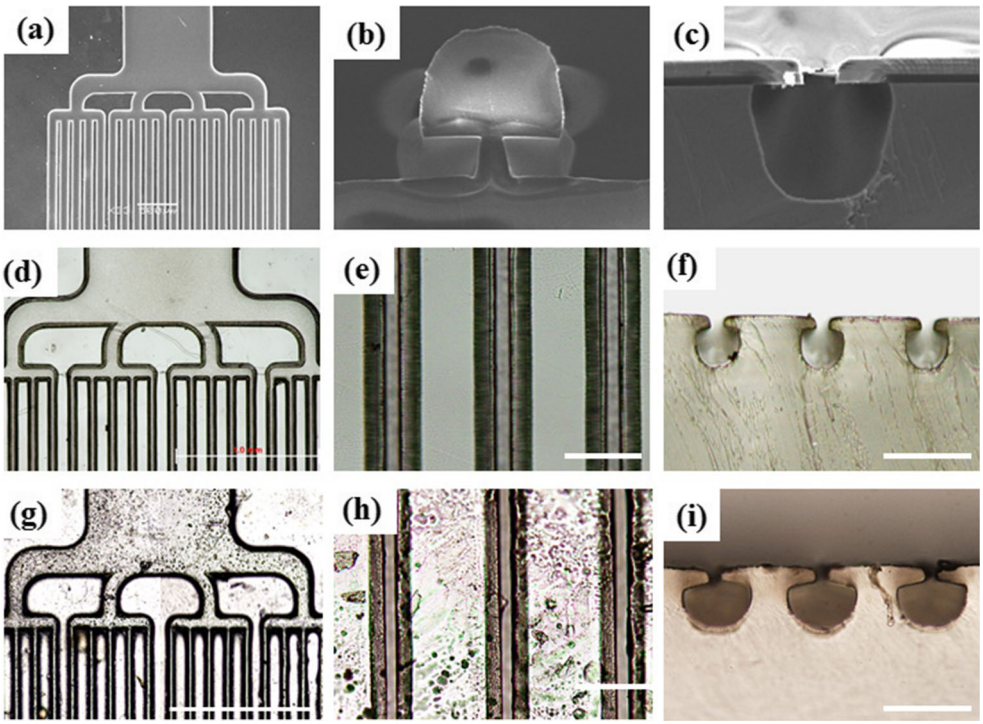

Fig. 5 Reentrant microvascular construct fabrication. **a** Top view and **b** cross sectional view of a silicon-photoresist mold. **c** A PDMS negative master mold. **d**, **e** Top views and **f** cross-sectional view of a PDMS reentrant microvascular construct. **g**, **h** Top views and **i** cross-sectional view of a PGS reentrant microvascular construct. The cross-sectional image illustrates near-cylindrical profile of the microchannels. Scale bars **a** 500 μm, **b**, **c** 20 μm, **d**, **g** 1 mm, **e**, **h** 50 μm, **f**, **i** 100 μm

reentrant microchannels with a depth of 35–80 μm. Cleft widths of 6–12 μm were fabricated, designed to be less than an endothelial cell diameter (d ~17 μm). An L/D aspect ratio of >550 (microchannel depth, D: 35–80 μm, microchannel length, L: 2 cm) could be achieved. The overhanging sealing wall thickness, defined by the initial photoresist thickness, was 8–15 μm.

The silicon-photoresist and PDMS negative molds displayed microchannels with a smooth profile at the base. The non-wetting of sharp corners in the PDMS master mold resulted in a near-cylindrical profile of the resultant reentrant microchannels.

A combination of anisotropic and isotropic etching also resulted in unity D/W aspect ratio microchannels.

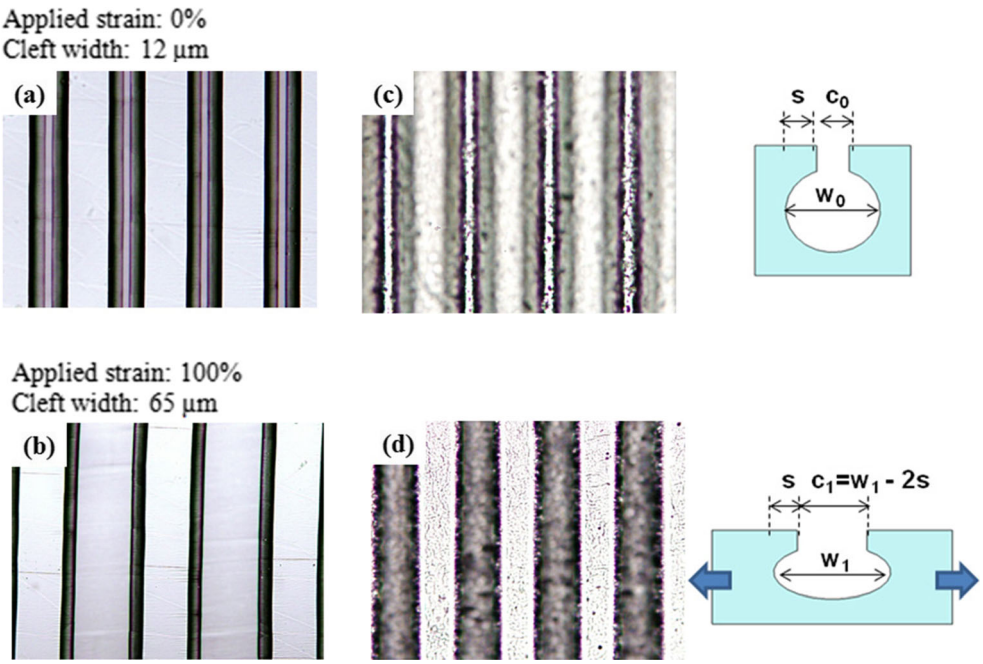

Fig. 6 Tensile loading of PDMS and PGS reentrant microvascular constructs and translation to cleft extension. Since the sealing wall width (s) remains constant, cleft extension, $((c_1 - c_0)/c_0)$ is greater than the applied strain $((w_1 - w_0)/w_0)$. PDMS construct from **a** to **b** and PGS construct from **c** to **d**, applied strain is 100 %, whereas cleft extension is 440 %

3.2 Endothelialization of the reentrant constructs

Strains up to 100 % could be successfully applied to the reentrant constructs without fracture. Since the width of the microchannel overhang on the sealing wall remained constant even upon application of a tensile load, the cleft extension, $((c_1-c_0)/c_0)$ was greater than the applied strain $((w_1-w_0)/w_0)$, as shown in Fig. 6. As an example, in the case of a network comprised of 50 μm microchannels, application of 100 % strain increased the cleft width from 12 to 65 μm; a cleft extension of ~440 % (Fig. 6a-d). Thus, given sufficiently elastomeric materials, cleft widths as small as 5 μm would be wide enough under applied tension to permit cell deposition.

Figure 7 illustrates endothelialized reentrant microvascular networks. Formation of confluent endothelial microchannels was observed within 1 day of cell seeding with panoramic imaging confirming spatially homogeneous cell seeding along the entire (2 cm) microvasculature length (Fig. 7a). Microchannels with a near-cylindrical geometry and diameters as small as 35 μm (~2× cell diameter) were fabricated and endothelialized. Cells bridged the cleft openings rendering the reentrant microvascular networks self-sealable for cleft widths smaller than the cells diameter. While constructs with 6 μm cleft widths were observed to self-seal without an additional seeding step, constructs with 12 μm cleft widths required an additional cell seeding step after release.

4 Conclusions

MEMS technology was applied towards the fabrication of a monolithic, elastomeric, near-cylindrical, reentrant microvascular network. PDMS was employed due to its high permeability, biocompatibility, and elastomeric nature (Borenstein

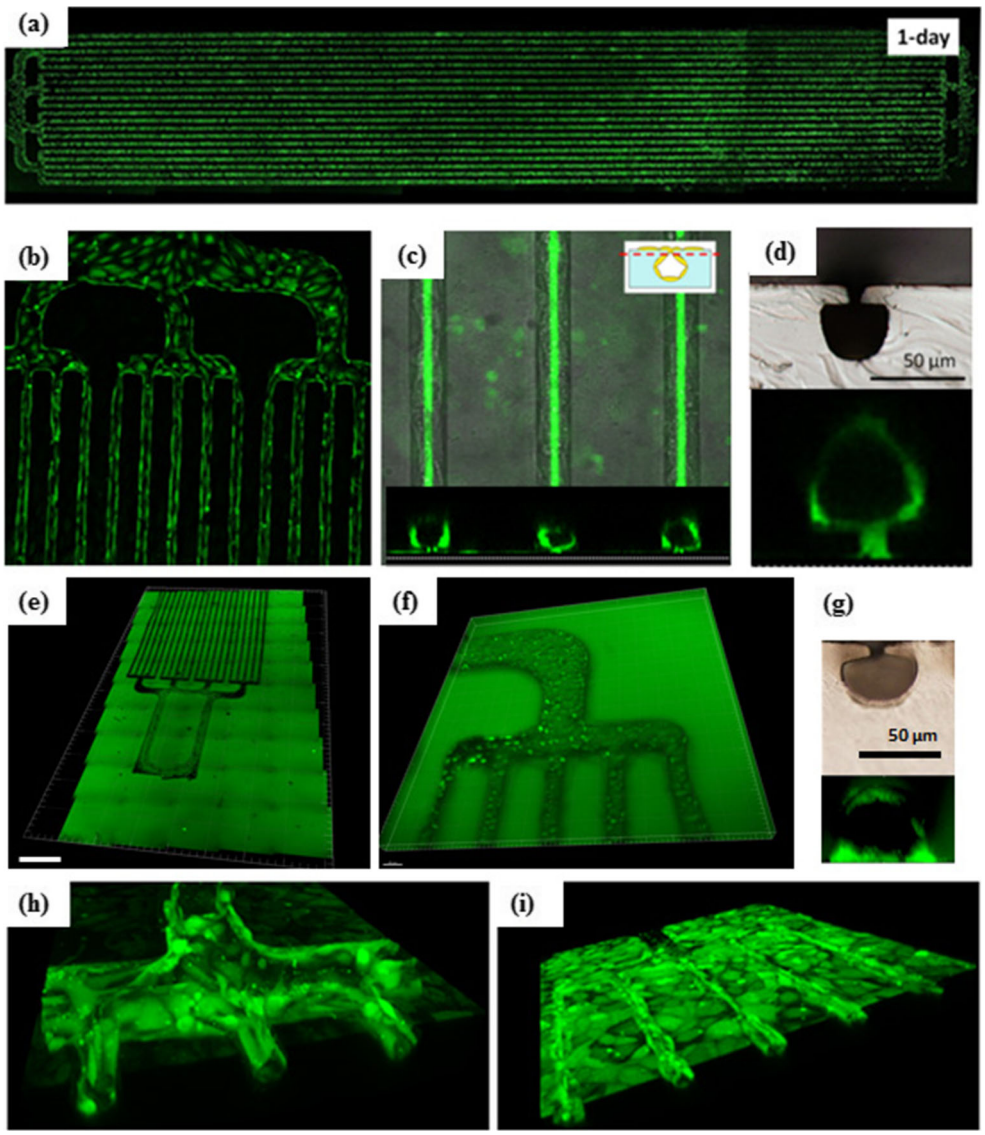

Fig. 7 Confocal microscope images of endothelialized reentrant microvascular constructs. **a** A panoramic image of a 2 cm long microvascular construct demonstrating the spatial homogeneity of the approach. **b** The base and **c** sealing walls of an endothelialized microvascular construct 1-day after initial cell seeding. **d** A cross sectional image of a 35 μm (~2 times cell diameter) wide reentrant microchannel with a cleft width of 6 μm. The cleft is self-sealed after endothelialization. **e**, **f** 3D reconstructions of endothelialized PGS reentrant microvascular constructs, **g** with the cleft self-sealed after endotheliazation. **h**, **i** 3D reconstructions of endothelialized PDMS reentrant microvascular constructs

et al. 2002; Shin et al. 2006). We utilized PDMS curing agent to pre-polymer base ratios of 1:10 and 1:5, which have been shown to mantain endothelial cell markers, viability, and proliferation (Esch et al. 2011; Lee et al. 2004). We also showed that this benign molding approach can be extended to a variety of other elastomeric biomaterials. PGS was chosen due to its biodegradable and biocompatible properties, as well as its broad application in microfluidics and tissue engineering (Wang et al. 2002; Wu et al. 2012). Endothelial cells grown on PGS exhibit normal cobblestone morphology, expressed von Willenbrand Factor (vWF), and mantained normal proliferation rate (Christina et al. 2005; Gao et al. 2007). Our simple molding and cell seeding approaches preserved biocompatible properties of PDMS and PGS, as indicated by normal endothelial cell morphology. The developed MEMS-assisted endothelialization approach allowed for spatially homogeneous endothelialization of high L/D (>550) microchannels with widths as small as 35 μm (d/D ~0.5). A high seeding density ($50–75\times10^3$ cells cm^{-2}) could be utilized to achieve confluent endothelial microchannels within 1 day.

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