Biomed Microdevices (2010) 12:71–79
DOI 10.1007/s10544-009-9361-1

Functional endothelialized microvascular networks with circular cross-sections in a tissue culture substrate

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Published online: 29 September 2009

Abstract Functional endothelialized networks constitute a critical building block for vascularized replacement tissues, organ assist devices, and laboratory tools for *in vitro* discovery and evaluation of new therapeutic compounds. Progress towards realization of these functional artificial vasculatures has been gated by limitations associated with the mechanical and surface chemical properties of commonly used microfluidic substrate materials and by the geometry of the microchannels produced using conventional fabrication techniques. Here we report on a method for constructing microvascular networks from polystyrene substrates commonly used for tissue culture, built with circular cross-sections and smooth transitions at bifurcations. Silicon master molds are constructed using an electroplating process that results in semi-circular channel cross-sections with smoothly varying radii. These master molds are used to emboss polystyrene sheets which are then joined to form closed bifurcated channel networks with circular cross-sections. The mechanical and surface chemical properties of these polystyrene microvascular network structures enable culture of endothelial cells along the inner lumen. Endothelial cell viability was assessed, documenting nearly confluent monolayers within 3D microfabricated channel networks with rounded cross-sections.

Keywords Microfluidics · Microfabrication · Endothelial cells · Vascular networks · Polystyrene

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1 Introduction

Progress in engineered replacement tissues and organs, advanced organ assist device technology, and drug development has been severely limited by an inability to establish functional microvascular networks with properties that mimic the physiology of the microcirculation (Vacanti and Langer 1999). Thick, complex tissues and organs require a viable microcirculation for delivery of oxygen and nutrients, while engineered tissues and organ assist devices demand high blood flow rates without significant endothelial activation or coagulation. Development of drugs for cardiovascular diseases and assessment of vascular toxicity for pharmacological entities requires the establishment of realistic *in vitro* models for the microcirculation as well.

The emergence of soft lithographic techniques for microfluidic devices (Duffy et al. 1998; Jo and Beebe 1999; Quake and Scherer 2000) and microscale fabrication technologies for tissue engineering (Bhatia and Chen 1999;

Desai 2000; Khademhosseini et al. 2006) over the past decade has spurred efforts to establish microvascular networks for both *in vitro* and *in vivo* applications. Microfluidic networks with controlled flow rates and levels of shear stress have been designed and constructed using PolyDiMethylSiloxane (PDMS), a biocompatible polymer that is the basis of many current microfluidic systems. These systems have been seeded with endothelial cells to demonstrate the concept of an intrinsic microcirculation for tissue engineering applications (Borenstein et al. 2002; Song et al. 2005) and with fibroblasts to demonstrate an *in vitro* adhesion assay (Lu et al. 2004). In addition, PDMS microfluidic constructs have been demonstrated for organ assist device applications in liver (Carraro et al. 2008; Leclerc et al. 2003), kidney (Kaazempur-Mofrad et al. 2004), and lung (Burgess et al. 2008). In each case, design of the microcirculation is aimed at maximizing oxygen and nutrient transport while minimizing the occurrence of coagulation due in part to the presence of disturbed flow. *In vitro* models using PDMS microfluidics have also been reported (Runyon et al. 2008) for use in investigating the mechanical and biochemical factors that contribute to blood clotting.

Common features of all of the aforementioned systems are that they are constructed using PDMS substrates, and that the microfabrication processes utilized to define the microchannels are not designed to produce cylindrical channels or smooth transitions at bifurcations or changes in diameter. These characteristics ultimately represent limitations for both *in vivo* and *in vitro* microvascular networks because of the non-ideal nature of the surface presented for cell seeding and the non-physiological fluid or blood flow through the network. The former limitation is related to the hydrophobic nature of the PDMS surface and the tendency for PDMS to adsorb and desorb molecular species in a transient and unpredictable manner. Significant efforts have been made to functionalize PDMS surfaces with various protein coatings and linker molecules to enhance the surface for cell culture (Hu et al. 2004), however, these approaches still suffer from the transient and poorly understood nature of the PDMS surface. This issue has been addressed by development of microfabrication techniques for microvascular networks in biodegradable polymers such as Poly(lactic co-glycolic acid) (PLGA) (King et al. 2004) and PolyGlycerol Sebacate (PGS) (Fidkowski et al. 2005). While these represent important potential avenues for vascularized tissue development, the biochemical nature of the surface and interaction with seeded cells is not well-understood nor is it stable over time. Therefore, a model system with well-known and stable surface properties would represent an important platform for *in vitro* devices and potentially for organ-scale assist devices as well.

Channel geometry and cross-section is the second feature that may limit the performance of existing microfluidic devices designed to mimic vascular networks. Typically, silicon masters for PDMS microfluidic channels utilize photolithographic patterns from SU-8 epoxy resin molds to form ridges with rectangular cross-sections. Even though these features may be rounded by the lithography process, the resulting PDMS microchannels have largely rectangular cross-sections as well, often with a single fixed depth and varying widths as defined by the lithographic pattern. Channel intersections and bifurcations may have sharp corners and sudden transitions in vessel dimensions, a result of the vertical walls in standard lithography processing. These geometries are readily fabricated in numerous polymeric substrates using conventional lithographic and molding techniques, but channel cross-sections are typically square, rectangular or trapezoidal.

The use of such microchannel cross-sections for microvascular networks and systems for blood flow is limited by numerous significant drawbacks. The resulting vessels and sharp corners are difficult to seed uniformly (Green et al. 2009), and are therefore inhospitable to the uniform stable cell seeding and formation of a confluent layer necessary to produce fully endothelialized microvascular networks. Importantly, shear stress controls numerous endothelial cell behaviors including their functional phenotype (Riha et al. 2005, Dai et al. 2004) and gene expression (García-Cardeña et al. 2001.) Wall shear stresses vary widely across the width of rectangular or trapezoidal microchannels (Lu et al. 2004), and therefore these geometries do not replicate either the microenvironmental cues necessary for vascular morphogenesis or for maintenance of physiologically stable and healthy microvascular networks.

In vitro models have demonstrated the importance of round channels and smooth transitions between microvessels for systems used for flowing blood. In work by Lima et al. (2008), experimental studies and theoretical modeling demonstrated the presence of anomalous behavior in the formation and size of the plasma layer of flowing blood in rectangular channels. The authors pointed towards the use of cylindrical microchannels with smooth transitions as a means to overcome these deficiencies but highlighted the challenges associated with the fabrication of three-dimensional microfluidic constructs with rounded channels. Coagulation studies (Shen et al. 2008) have shown that geometry plays an extraordinarily important role in the coagulation process, and extensive theoretical modeling (Runyon et al. 2008) has provided a foundation for understanding these phenomena in a quantitative manner.

These studies all point to the necessity for microfluidic structures with rounded channels and smooth transitions to

support microvasculatures seeded with confluent, functional endothelial cells. Recently there have also been several reports of rounded structures in silicon wafers and polymer scaffolds designed to replicate microvasculature. One approach entails the use of photoresist-based molds in which the resist profiles are rounded by heating after the exposure and development steps (Wang et al. 2007). Silicon masters with rounded photoresist profiles were used to generate PDMS replicas, and the PDMS half-channels were joined using alignment techniques to form cylindrical luminal structures, prior to seeding with endothelial cells. However, this approach does not enable precise control over the transitions between channels of different diameters and does not enable smoothening of channel bifurcations and intersections, and therefore the smooth, rounded nature of physiological vasculature is not fully replicated. In another report (Seo et al. 2004), rounded photoresist was combined with isotropic etching to create cylindrical structures in silicon, but again the opportunity for smooth and gradual transitions at intersections and vessel diameter changes was limited. Camp and co-workers (Camp et al. 2008) demonstrated the use of xenon difluoride etching to generate semi-circular channels in silicon wafers and produced networks with varying channel radii. These half-channels were seeded with endothelial cells as a demonstration of the capability of the technology.

In this report we describe a new technique enabling the generation of microfluidic networks that mimic the geometry of the vasculature with circular channels, smooth bifurcations and gradual transitions between vessels, constructed in the well-characterized substrate, commonly used for tissue culture. The new platform we propose for fabricating microfluidic scaffolds for tissue engineering is based upon a combination of master molding of structures that mimic the geometry of microvascular networks, and hard embossing of these structures into polystyrene. The overall strategy is to utilize this precise microfluidic fabrication technology, along with an endothelial cell model system, to clearly establish an endothelial functional phenotype in a true 3D construct. This enabling technology will serve as a platform for future work in which this system will be translated into precision 3D biodegradable scaffolds integrated with co-culture models and matrix materials suitable for tissue engineering applications. This master molding process has been demonstrated to produce cylindrical geometries suitable for microvessels at diameters as small as tens of microns, distinguishing it from other common microfluidic fabrication techniques such as SU-8 photolithography or Deep Reactive Ion Etching. The basic molding technique was pioneered by LaVan, George, and Langer (LaVan, et al. 2003), in which polypyrrole and nickel were electrodeposited to form master molds with the inverse pattern for subsequent replica molding. We have modified this technology using a Cu electrodeposition system and have established nearly perfectly inverse circular geometries in master molds for subsequent hard embossing into tissue culture grade polystyrene. These structures have been seeded with human endothelial cells to form confluent intraluminal layers and cell viability has been demonstrated over periods of 24–48 h.

2 Methods

For the initial demonstration of this technology, we chose a simple bifurcated microchannel network and utilized two different types of patterns, one with constant vessel height and another with the capability to vary the channel diameter between different generations of vessels. Wafers were electroplated with copper to form semi-circular ridges on the silicon substrate; these master molds were used to emboss thin polystyrene sheets with semi-circular channels. Once the sheets are embossed, they are joined with alignment techniques to form closed microchannel networks suitable for seeding endothelial cells. Human umbilical vein endothelial cells (HUVEC) were seeded onto the inner surface of the cylindrical channel networks, achieving confluence, and the cells were exposed to shear flow and stained to demonstrate functionality with calcein AM stain.

2.1 Electroplated silicon master mold: single diameter design

For the simple 2D microchannel network, a CAD layout was generated using Tanner L-Edit for a simple bifurcated network with channels of channel diameters of 200 microns and above. The electroplating process used to create these simple bifurcated networks is known as the “buried electrode” process. The buried electrode begins with a blanket metal layer of chrome/gold (1000/3000 Å) sputtered on a silicon wafer. Silicon dioxide is then deposited on the seed-metal using plasma-enhanced chemical vapor deposition (PECVD; Oxford System 100 PECVD Reactor) to a thickness of 0.5–1.0 μm. The silicon dioxide is then patterned using contact lithography (Karl Suss MA-6) with 2 micron thick photoresist (Shipley 1822 photoresist) and developed in standard developer solution (MF319), followed by wet-etching of the oxide in hydrofluoric acid (Buffered Oxide Etch DI 1:7 HF) and photoresist removal with a solvent (SVC-14). With the seed metal exposed only where the oxide has been removed using the lithographic patterning, electroplating with copper then follows. Copper is electroplated by submerging the wafer in a bath solution (Technic, Inc. Copper Bath RTU) at a current of 70 mA, resulting in a copper growth rate of 15 μm/hr.

2.2 Electroplated master molds with multiple channel diameters and smooth transitions

The electroplating process for bifurcated channel networks in which the diameter is varied between generations is known as the "patterned gap" process. The principle behind the patterned gap process relates to the fact that electroplated structures will begin to form first on seed metal patterns that are electrically connected to the plating electrodes. These seed metal structures will result in semicircular raised structures as described above, but electroplating of adjacent seed metal patterns separated by a gap or spacer will not begin to form immediately. Once the electroplated structures have expanded towards the patterns separated by a gap, as shown in Fig. 1(b), electroplating will begin on these separated seed metal patterns as electrical connection is established.

For the patterned gap process, wafers receive a blanket layer of thermal silicon dioxide to isolate the silicon from the seed-metal. The thickness of this thermal oxide is again between 0.5 and 1.0 μm thick. The seed-metal layer is formed using a lift-off process. The'lift-off" resist (Microchem Corp. LOR series) is deposited on the oxide surface first, followed by the second level resist (AZ 1518, Microchem. Corp.), and the resist stack is patterned using a contact aligner (Karl Suss MA-6). The 1518 is developed using diluted AZ400K developer and the LOR using MF319. Once the photoresist has been patterned, the seed metal (1000 Å Cr/ 3000 Å Au) is sputter-deposited (Mill Lane DC Magnetron Sputterer) and liftoff is achieved via ultrasonic agitation in solvent. Copper is electroplated is by submerging wafers in a commercially-available bath (Technic, Inc. Copper Bath RTU) and applying 70 mA to achieve a copper growth rate of 15 μm/hr.

2.3 Polystyrene layers with embossed round channels

Polystyrene (PS) sheets from Plaskolite, Inc. (040" Clear UVF Styrene) were cut to size and hot embossed using the copper-plated silicon mold. The silicon mold and polystyrene sheet were sandwiched between two pieces of Kapton film (Fralock Inc.) in a pressure vessel; temperature was raised to 130°C, and the chamber evacuated, causing the piston of the vessel to exert a pressure of 25 in. Hg. After heating the mold/PS composite structure for 15 min at 130° C, the apparatus was cooled to ~40°C. The mold and substrate were then removed from the embosser and separated to form a free-standing polystyrene device with half-cylindrical microchannels.

2.4 Polystyrene layer bonding

Two techniques were developed for bonding the PS layers together. In the first method, two embossed polystyrene parts were treated with oxygen plasma (Prichard et al. 2007) using a Technics Microstripper, with a setting of 100 W and 100 mTorr O_2 pressure for 1 min. The treated surfaces were placed in contact, and the channels were aligned with the aid of an optical microscope. Flexible silastic tubing (Dow Corning Inc.) was cut and placed in the deeper channels for tubing connections. The two layers were thermally bonded in the embossing apparatus at 90°C for 30 min. The flexible tubing was sealed in place during bonding.

In order to achieve finer alignment of the half-cylindrical channels, a second bonding technique was also utilized. Channels were aligned using a modified optical aligner (HTG-IV Contact Aligner, Hybrid Technology Group). The embossed polystyrene pieces were exposed to oxygen plasma at 100 mW and 100 mTorr for 1 min to activate the surfaces, and then affixed to a glass mask plate and a glass wafer with PDMS. The mask plate and wafer were then placed in the aligner, and structures were aligned using the x, y, and θ micrometers; alignment was evaluated optically with the microscope. A small amount of urethane adhesive (Double/Bubble Urethane, Elementis Specialties, Inc.) was applied to the outer edges of the polystyrene pieces, away from channels, and pieces were brought into

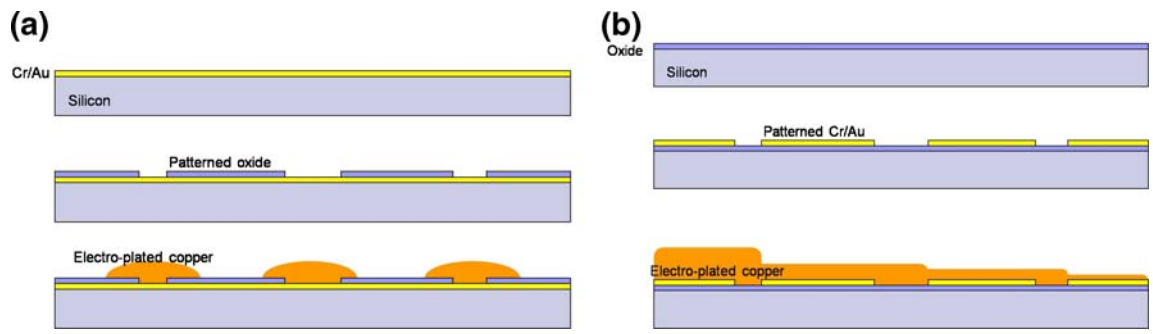

Fig. 1 (**a**) Schematic of Cu electroplating process starting with blanket seed metal deposition, patterning of a low-temperature oxide, and electroplating of copper on open areas of seed metal and expanding isotropically as the plating time is increased. (**b**) Schematic of Cu electroplating process for microchannel networks with varying diameters, in which the seed metal is patterned so that subsequent electroplating contacts new open seed metal areas after a time delay, thereby controlling the feature height and width

contact. After curing for 1 h, polystyrene pieces were removed from the glass substrates and placed between pieces of kapton film in the heated pressure vessel. The chamber was heated to 95°C, and a pressure of 40 psi was applied for 30 min. Tubing was connected to the bifurcated channel networks, and the devices were sealed with silicone sealant to enable endothelial cell seeding and culture.

2.5 Human endothelial cells

Primary human umbilical vein endothelial cells (HUVEC) were isolated and cultured, as previously described (García-Cardeña et al. 2001). HUVEC were cultured in Medium-199 (BioWhittaker), supplemented with 50 μg/ml endothelial cell growth supplement (Collaborative Research), 100 μg/ml heparin (Sigma), 100 units/ml penicillin plus 100 μg/ml streptomycin (BioWhittaker), 2 mM L-Glutamine (GIBCO), and 20% FBS (BioWhittaker). These cells were then plated on 0.1% gelatin (Difco)-coated circular channels, and maintained at 37 C and in 5% CO_2 for the duration of the experiments.

2.6 Endothelial cell adhesion and viability

The viability of primary cultures of HUVEC within the 3D network was determined using calcein AM live cell stain according to the manufacturer's instructions (Invitrogen). Briefly, HUVEC were washed once with DPBS and then incubated at room temperature with 4 μM Calcein AM, diluted 1:1000 from the manufacturer's solution of 4 mM in DPBS, for 30 min. Following the incubation, cells will be washed once with DPBS and then maintained in the medium described above. In order to evaluate HUVEC adhesion and viability within the closed channel circular networks, both phase contrast and fluorescent images (485 nm excitation wavelength) was obtained with a Nikon Eclipse TE300 inverted microscope (Nikon) at 20X and 4 X magnifications, respectively, 24 h after plating.

3 Results and discussion

3.1 Buried electrode and patterned gap electroplating

For the simplest case, with a single height of microchannels, a simple bifurcated network with the minimum channel diameter of 200 microns was produced on the master mold wafer. The aim of this experiment was to demonstrate the ability of the electroplating process to produce half-cylindrical raised geometries. The "buried electrode" electroplating (Fig. 1(a)) was used to produce 2D microchannel networks with rounded cross-sections for hard embossing of polystyrene layers. Blank silicon wafers were sputtered with seed metal, and then oxidized using a chemical vapor deposition process. The oxide was then lithographically patterned to expose areas for Cu electrodeposition.

In a second experiment, the copper electroplating process known as the "patterned gap" process, capable of producing silicon master molds with a range of microchannel depths and smooth flow transitions at bifurcations and between successive generations of channels, was used to produce more complex geometries on silicon wafers. Both the buried electrode and the patterned gap processes possess the fundamental capability to produce networks of channels with varying diameters, but the patterned gap process offers much more flexibility in generating smooth, rounded structures with gradual transitions at diameter changes and intersections. The patterned gap process, shown in Fig. 1(b), had a blanket layer of thermal oxide roughly 1.0 microns in thickness to isolate the silicon from the seed-metal. The seed metal comprised 1000 Å Cr and 3000 Å Au, formed using a specialized 'lift-off' photoresist process. Following seed-metal formation, wafers were electroplated with copper by submerging them in a commercially-available bath solution; this process produced a reliable copper growth rate of 15 μm/hr.

The Cu electroplating began immediately in regions for which the seed-metal pattern is directly connected to the anode, but is delayed in regions separated from the anode until enough material has plated onto the wafer to bridge the patterned gaps. The length of these gaps governs the delay time prior to the start of Cu formation onto the next level of microchannels with the network design, allowing for structures of varying cross-section to be created. For instance, a gap of 100 microns introduces a time delay such that the largest ridges are 100 microns taller than the next level structures. By varying the gaps in a systematic fashion an entire range of microvessel diameters may be achieved on embossed layers. An image of an electroplated mold with eight generations of bifurcated microchannels with semi-circular cross-section is shown in Fig. 2, with five generations shown in the SEM in Fig. 2(a) and a close-up of the bifurcation points shown in Fig. 2(b). The elliptical cross-sections have a minor axis radius of 80 microns and a major axis diameter of 190 microns, and the bifurcation points shown smooth transitions that allow non-disturbed flow through those regions.

3.2 Polystyrene constructs for cell culture

Establishment of a robust functional phenotype for endothelial cells in 3D microfluidic constructs requires exquisite control over numerous parameters associated with the cell microenvironment. Among these are surface chemical interactions between the cultured endothelial layer and the

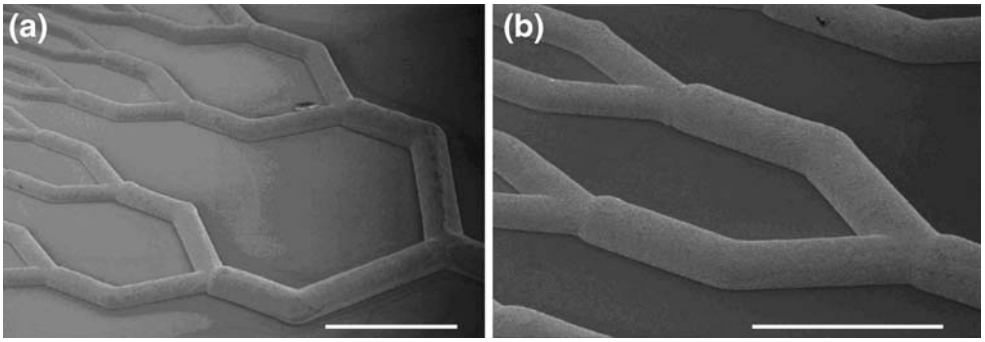

Fig. 2 (**a**) SEM image of a Cu electroplated mold on silicon wafer showing four levels of smooth vessel bifurcations. (**b**) Higher magnification SEM image showing inverse half-cylindrical geometry of the electroplated mold to be used to emboss half-cylindrical channels in polystyrene. Scale bars are 1 mm (*a*) and 500 microns (*b*)

walls of the scaffold, and the stability of the surface over time. Therefore, we have chosen tissue culture grade polystyrene sheets as the platform for the development of 3D structures for cell seeding and tissue engineering. Tissue culture-grade polystyrene is a well-established platform for culture of numerous cell types, and serves as the industry standard for cell and tissue arrays for high-throughput screening and other laboratory applications. Processes for micromolding of polystyrene have been established (Heckele and Schomburg 2007) and several groups have reported successful molding of polystyrene and polycarbonate for cell culture and tissue engineering applications (Heckele and Schomburg 2007; Giselbrecht et al. 2006; Hu et al. 2005). We have established a robust, reproducible and high-precision hard embossing technique in which Cu-electrodeposited master molds are used to form half-cylindrical features and smooth bifurcations and intersections in microfluidic vessel network structures.

To generate the microfluidic structures, polystyrene sheets were hot embossed using the copper-plated silicon mold described above. The silicon mold and polystyrene sheet were sandwiched between two pieces of Kapton film under controlled temperature and pressure conditions, at a maximum temperature of 130°C. The mold and substrate were then removed from the pressure vessel and separated to form a free-standing polystyrene device with rounded microchannels. Deeper channels that allow for incorporation of flexible tubing were created by attaching cross-sections of metal tubing to the silicon mold at the ends of the microchannels. An example of an embossed polystyrene part is shown in Fig. 3(a) and (b).

3.3 Polystyrene layer bonding to form closed circular microchannels

Two embossed polystyrene parts were treated with oxygen plasma to create a more hydrophilic surface friendly to cell adhesion (Walboomers et al. 1998). The treated surfaces were placed in contact, and the channels were aligned with the aid of an optical microscope. Flexible silastic tubing was cut and placed in the deeper channels designed for tubing connections, and the two layers were thermally bonded in the embossing apparatus. A cross-section of the bonded structure, showing a round channel approximately 175 microns in diameter, is shown in Fig. 3(c). The bond interface is not visible; perhaps in part due to the surface polishing of the sample, but also indicative of an excellent seal. The alignment between the two half-channels is quite good, to within less than 10 microns of misalignment. There is no visible evidence of channel deformation or collapse due to the bonding process.

3.4 Endothelial cell culture in cylindrical microchannel networks

Critical to developing a 3D endothelial network is the ability for endothelial cells to attach, and grow as a confluent monolayer on the network channel walls and maintain viability under normal culture conditions. Thus, we simultaneously assessed cell attachment and biological compatibility of the newly fabricated system by culturing primary human umbilical vein endothelial cells (HUVEC) within the circular channel networks. As seen in Fig. 4, cell attachment and monolayer formation within polystyrene networks was achieved and it was best visualized using phase contrast imaging (Fig. 4(a)), while cell viability was confirmed using a calcein AM live cell stain in which only viable cells fluoresce (Fig. 4(b) and (c)). Both cell attachment/monolayer formation and viability were consistent among channels and independent of the channel diameter. These data demonstrate that human endothelial cells attach, form a confluent monolayer, and remain viable for at least 24 h of culture within the 3D networks manufactured with this new technology.

3.5 Discussion

The development of *in vitro* models as well as direct therapeutic devices such as engineered tissue constructs and artificial organ assist devices has been limited by the inability to produce engineered vascular networks that mimic physiological properties. To this point, microfluidic

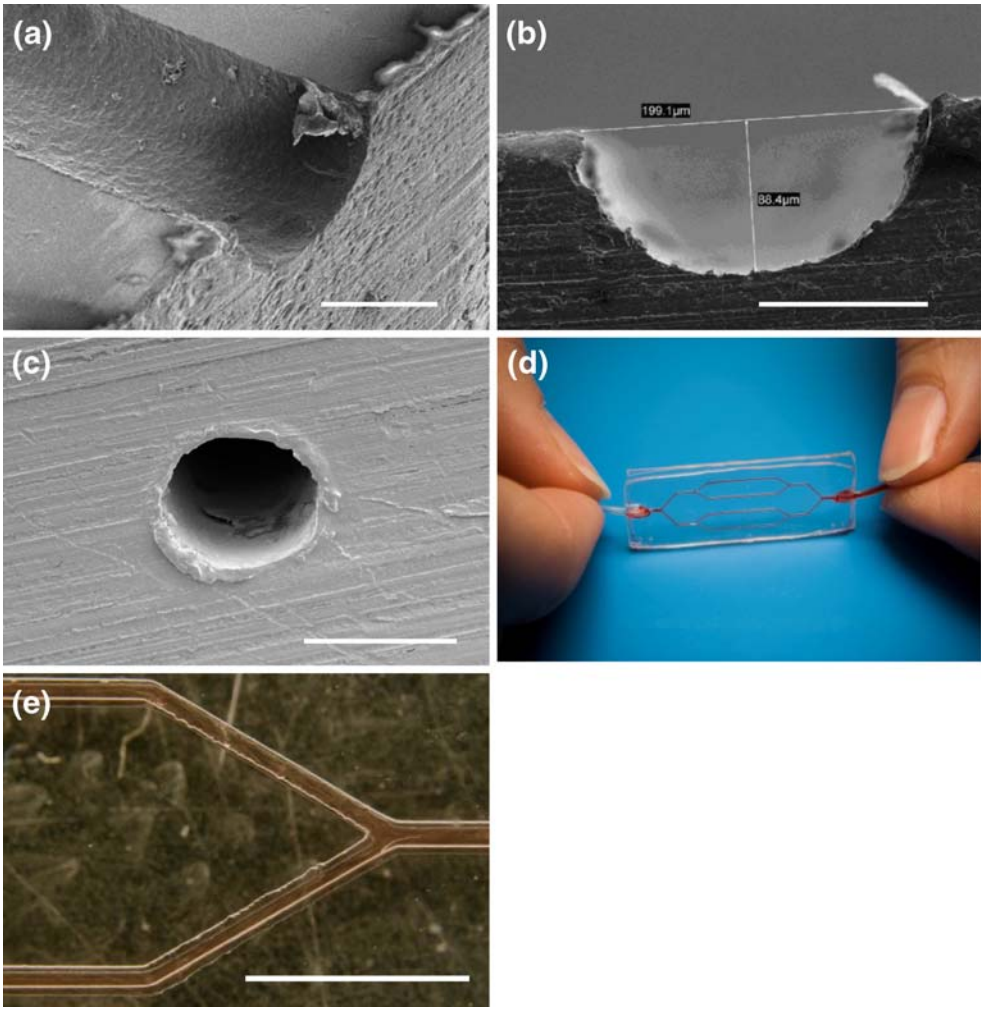

Fig. 3 SEM image of polystyrene film hard-embossed using a Cu electroplated mold: (**a**) Top view and (**b**) side view. (**c**) Sealed polystyrene channel cross-section, showing circularity and absence of bond interface. (**d**) Photo of device with integrated tubing. (**e**) Optical micrograph of bifurcation region. Scale bars: 100 microns (*a*, *b*); 200 microns (*c*); 2 mm (*e*)

structures used for both lab-on-a-chip systems and for artificial tissues and organs have relied upon traditional PDMS-based fabrication techniques, using replica molding of PDMS sheets from silicon master molds formed using conventional photolithography. These PDMS-based microfluidic networks have limited utility for two reasons: 1) The surface chemistry of the PDMS fluidic networks is not stable and does not replicate the chemistry of the basement membrane of blood vessels, and 2) PDMS replica molding results in rectangular channel geometries with sharp transitions between vessel intersections and difficulty in varying the height of microchannels along with the width.

These limitations result in serious performance problems with PDMS-based microfluidic systems, including difficulties in obtaining strongly adherent endothelial cells on the sidewalls of devices, particularly in a high shear stress environment, challenges associated with seeding endothelial cells to form confluent luminal structures in the presence of rectangular walls and corners, and non-physiologic blood flow conditions at sudden expansions, contractions and sharp intersections. Difficulties in cell seeding will ultimately limit the accuracy of laboratory-on-a-chip systems in producing phenotypic uniformity in the presence of fluid mechanical forces and chemical signals, due to the non-uniform spatial distribution and exposure of endothelial cells to these signals in the engineered constructs. Non-optimal blood flow conditions due to geometric factors in microfluidic constructs leads to increased coagulation and inflammatory responses, a major problem in artificial organ assist devices.

The microfabrication technology reported here enables many of these difficulties and limitations in conventional microfluidic systems to be overcome. The use of tissue-culture-grade polystyrene provides a well-understood and stable surface chemistry for cell attachment, as this is the basis of standard well plate systems. For some applications, tissue culture plastic may not have the desired material properties; the fabrication techniques described herein are broadly applicable to the formation of round channels in PDMS and a host of other substrate materials. Round channels with smooth transitions and intersections enable uniform cell seeding and exposure to consistent levels of

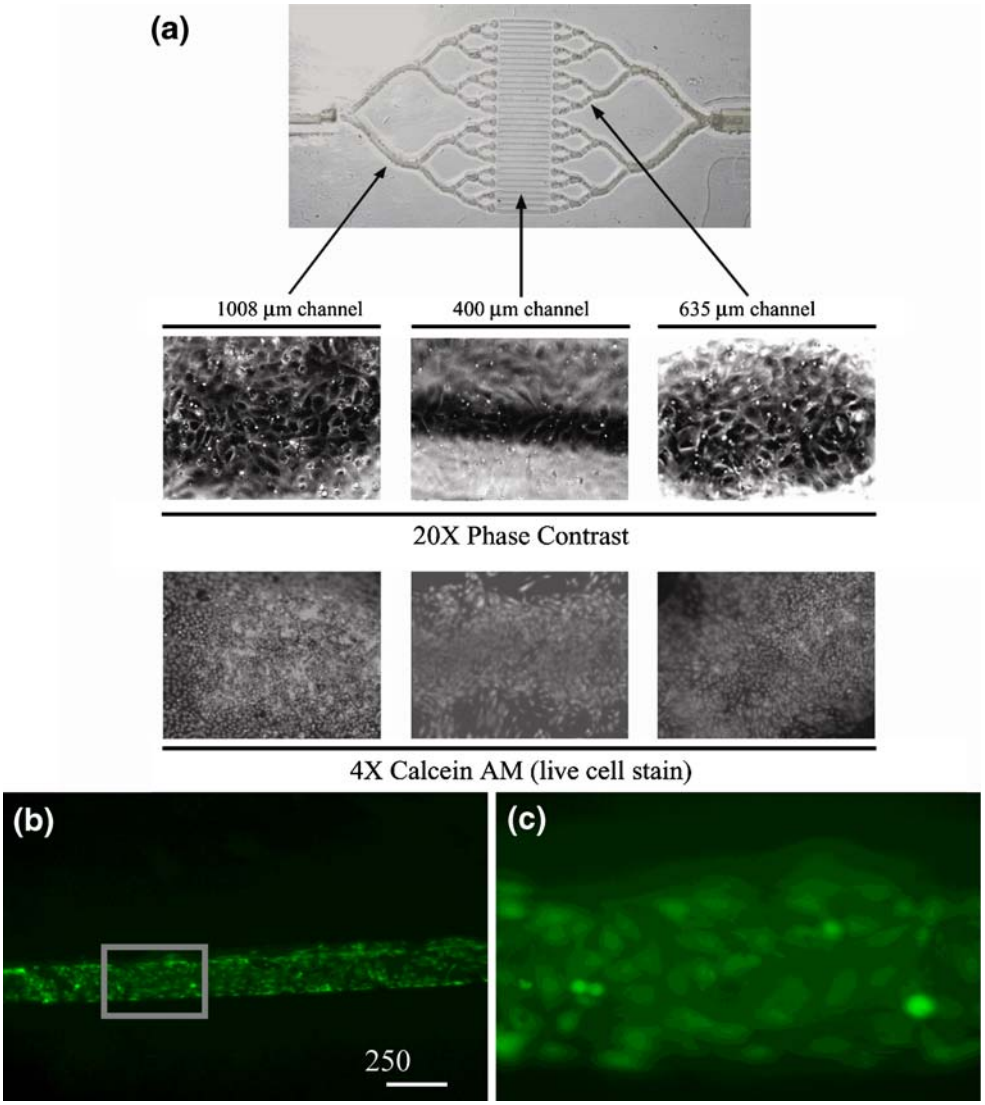

Fig. 4 Human endothelial cells were cultured in circular polystyrene channels, generated with the fabrication process described in the text. (**a**) Diagram showing cell adhesion and monolayer formation for primary endothelial cells seeded in a closed channel, 3D printed, polystyrene network. (**b**) and (**c**) Cell viability was confirmed after 24 h of culture, via calcein AM live cell staining. (**b**) 4X and (**c**) 20X magnification, with (*c*) corresponding to the outlined region in (*b*)

fluid mechanical shear and other signals within the microvascular networks. Anomalies associated with the flow of culture media and blood in these structures are also avoided, a major advantage of these systems relative to current technology. The specific designs used for microvascular networks with multiple channel diameters and smooth transitions can be selected across a wide range of parameters. For instance, Murray's Law, which invokes minimum work considerations, holds that the sum of the cubes of the diameters of the two daughter vessels at a bifurcation should equal the cube of the diameter of the parent vessel (Lim et al. 2003.) The bifurcation angle is governed by additional considerations regarding smooth blood flow and effective distribution of oxygen and nutrients to surrounding tissues. In the designs utilized here, generalized network design approaches meant to demonstrate the ability of the fabrication technology to produce microvessels with biomimetic features guided by the above principles were utilized. Future work is planned for which designs based on the specific principles of vessel development and oxygen and nutrient distribution as guided by the abovementioned principles will be generated.

The principal challenge associated with the current fabrication technique is related to precise alignment of the two half-channel surfaces; this issue has been addressed separately (Marentis et al., IEEE JMEMS 2009). Other challenges are associated with the high degree of substrate stiffness of polystyrene relative to basement membrane, which is a common problem in cell-based assays in conventional well plates, and the fact that polystyrene is a rigid, permanent structure not suitable for scaffolds and implantation for tissue engineering applications. These challenges may be addressed by the development and substitution of soft, elastomeric, biodegradable yet stable substrates for applications using this microfabrication technology for fabrication of vascular networks.

4 Summary

Microfluidic systems used to produce microvascular networks for *in vitro* laboratory systems and therapeutic devices such as engineered tissue constructs and organ assist devices are currently limited by geometric and surface chemical considerations associated with available technologies. In this report, we have described a new technique for producing round channels and smooth fluidic transitions in bifurcated networks in order to better replicate the flow properties and physiology of the microcirculation. These structures have been built in polystyrene, a well-characterized and commonly used material for cell culture and cell-based assays. Bifurcated microchannel networks comprising rounded channels with smooth fluidic transitions have been constructed in tissue culture grade polystyrene, as a proof of concept for the development of microvasculature. These constructs have been seeded with primary cultures of human endothelial cells, which have been cultured to confluence, and viability has been assessed using a calcein AM stain to demonstrate viable endothelium in a physiologically relevant fluid dynamic and surface chemical microenvironment for the first time. This advance has important implications for both *in vitro* model systems and therapeutic devices such as organ assist systems and engineered tissue constructs. Future directions include the translation of this technology into substrates that have mechanical properties more closely resembling basement membrane, and ultimately into biodegradable scaffolding materials more suitable for implantation.

Acknowledgements The authors would like to acknowledge the MEMS Technology Group at the Draper Laboratory for fabrication of master molds and use of facilities, and funding provided by Charles Stark Draper Laboratory and Brigham and Women's Hospital.

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