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Endothelialized Networks with a Vascular Geometry in Microfabricated Poly(dimethyl siloxane)

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Abstract. **One key challenge in regenerating vital organs is the survival of transplanted cells. To meet their metabolic requirements, transport by diffusion is insufficient, and a convective pathway, i.e., a vasculature, is required. Our laboratory pioneered the concept of engineering a vasculature using microfabrication in silicon and Pyrex. Here we report the extension of this concept and the development of a methodology to create an endothelialized network with a vascular geometry in a biocompatible polymer, poly(dimethyl siloxane) (PDMS). High-resolution PDMS templates were produced by replica-molding from micromachined silicon wafers. Closed channels were formed by bonding the patterned PDMS templates to flat PDMS sheets using an oxygen plasma. Human microvascular endothelial cells (HMEC-1) were cultured for 2 weeks in PDMS networks under dynamic flow. The HMEC-1 cells proliferated well in these confined geometries (channel widths ranging from 35 μm to 5 mm) and became confluent after four days. The HMEC-1 cells lined the channels as a monolayer and expressed markers for CD31 and von Willebrand factor (vWF). These results demonstrate that endothelial cells can be cultured in confined geometries, which is an important step towards developing an *in vitro* vasculature for tissue-engineered organs.**

Key Words. **tissue engineering, replica molding, biocompatible polymer, endothelialization**

Introduction

Since the first conception of tissue engineering as an approach to alleviate donor organ shortage and provide an alternative treatment for tissue and organ failure, significant progress has been made towards that goal, and transplantation of selected cell populations has become a major approach (Langer and Vacanti, 1993; Griffith and Naughton, 2002). It is generally accepted that the regeneration of quasi-two-dimensional (2-D) organs, e.g., skin or bladder, and avascular tissue, e.g., cartilage, is closer to clinical applications than the regeneration of thick, three-dimensional (3-D) organs. Tissue-engineered skin and cartilage are either already on the market or in clinical trials (Naughton and Mansbridge, 1999; Fauza, 2003).

One of the key challenges in the regeneration of vital organs, e.g., liver and heart, concerns the transport requirements for transplanted cells. To meet the metabolic needs for nutrient and oxygen delivery and waste removal, transport by diffusion is no longer sufficient but requires a convective pathway, i.e., a vasculature, among the transplanted cells. Relying on host vascularization alone is not sufficient, and one approach towards establishing a vasculature is to enhance angiogenesis through delivery of growth factors (Tabata et al., 1999; Peters et al., 2002; Perets et al., 2003).

Our laboratory recently described a novel approach for engineering as vasculature *in vitro* (Kaihara et al., 2000). The methodology is based on semiconductor wafer process technology originally developed for integrated circuits (IC) and microelectromechanical systems

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(MEMS). Silicon microfabrication offers submicron-scale resolution over several orders of magnitude from 0.1 μm to 10's of cm (Madou, 2002). Since this range covers the relevant physiological length scales from capillaries to large vessels, a concept was developed to create a complete branching vascular circulation in 2-D on silicon wafers and subsequently build up 3-D structures by stacking or rolling. In a first demonstration, hepatocytes and endothelial cells were cultured on patterned silicon and Pyrex wafers. Hepatocyte sheets were lifted off, folded into compact 3-D configurations and implanted into rat omenta, resulting in the formation of vascularized hepatic tissue (Kaihara et al., 2000).

Silicon microfabrication has also become a useful tool in cell biology for spatial control of cells in culture. Ongoing efforts focus on the design of material structures that lead to controlled or predictable cellular response (Curtis and Wilkinson, 1998; Teixeira et al., 2003). Whitesides' seminal work in the development of various related processes, collectively termed "soft lithography", has allowed the fabrication of soft materials with precise geometric control (Whitesides et al., 2001). These patterned soft materials have found application in diverse areas such as cell patterning and microfluidic devices for cell sorting (Folch and Toner, 2000; Pins et al., 2000; Quake and Scherer, 2000; Wu et al., 2003).

Here we report an extension of the original concept and the adoption of soft lithography to demonstrate the feasibility of forming a network with a vascular geometry in a biocompatible polymer. A vascular network was designed by a computational model that approximates physiologic blood flow and accounts for the rheological properties of blood. This model is a tool to optimize the design of the vascular networks to provide an even distribution of blood across the entire network with minimized resistance to blood flow while providing maximum mass transfer and oxygen to the surrounding tissue. Using photolithography, master molds were fabricated by etching the network pattern into silicon wafers. Closed channels were created from silicon master molds by replica molding of poly(dimethyl siloxane) (PDMS) and subsequent plasma-bonding of a patterned PDMS template to a flat PDMS sheet. Human microvascular endothelial cells (HMEC-1) were cultured in these confined geometries and characterized using optical microscopy and immunohistochemistry.

Materials and Methods

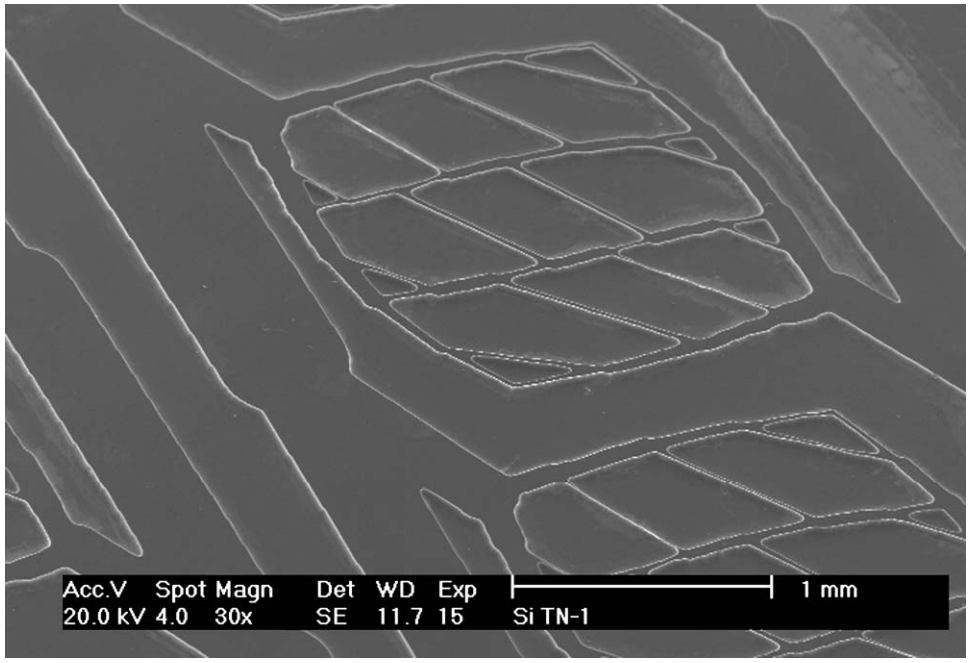
Silicon microfabrication

The network resembling a capillary bed was designed using a computational algorithm for the simulation of blood flow in vascular networks. This model accounts for the non-Newtonian rheology of blood and simulates the hematocrit, flow rate and pressure. It also emulates adaptation and remodeling of the vascular structure in response to deviation from homeostatic shear stress levels, thereby allowing for dynamic evolution of the network design (Kaazempur-Mofrad et al., 2001). The network used in this study has a total surface area of 13.69 cm^2 and an internal volume of 21.3 mm^3. The widths of the vessels range from 35 μm to 5 mm, and the channel depth is uniformly 35 μm.

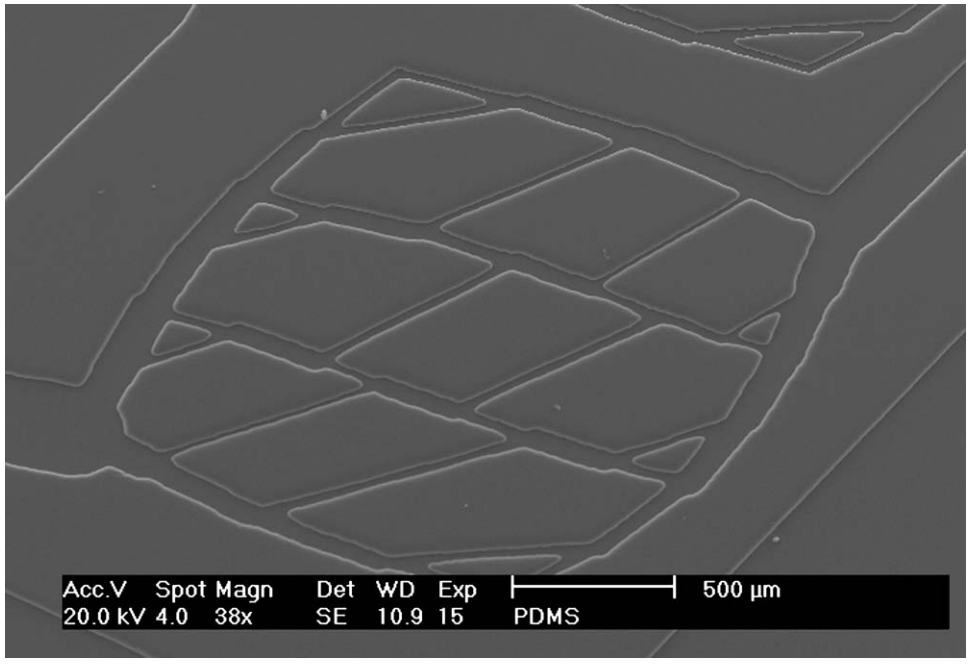
The network design was transferred to a mask layout editor (Tanner Research, Inc., Pasadena, CA), and a photolithographic mask was made using a high-resolution line printer (PageWorks, Cambridge, MA). Standard photolithography was employed for wafer processing. A 10 μm thick film of photoresist (Shipley AZ4620, MicroChem, Newton, MA) was spin-coated on clean silicon wafers. Following a pre-bake, the photoresist was exposed to UV light using a mask aligner (Karl Suss MA-6; Suss America, Waterbury, VT). The exposed wafers were developed (Shipley AZ400K) and baked. The network pattern was etched using reactive ion etching (Surface Technology Systems, Newport, UK). The remaining resist was stripped with acetone, and the wafers were cleaned with sulfuric acid and hydrogen peroxide. To faciliate mold release for polymer replica molding, the etched wafers were passivated in an inductively-coupled plasma with C_4F_8. Figure 1(a) shows a scanning electron microscopy (SEM) image of the etched silicon wafer.

Micropatterning of biocompatible polymer

The etched wafers were used as master molds for replica molding of PDMS. To achieve a seamless connection between the network and tubing, 5 mm long segments of Teflon tubing (1/8 inch outer diameter (OD), 1/16 inch inner diameter (ID); Cole-Parmer, Vernon Hills, IL) were placed on the wafer as spacers for the inlet and outlet and temporarily secured with cyanoacrylate glue. A thin coating of PDMS (Sylgard 184, Dow Corning, Midland, MI) was cast and cured at 65°C for 4 hours. After curing was complete, 10 mm segments of silastic tubing (OD = 1/4 inch, ID = 1/8 inch, Cole-Parmer) were placed over the Teflon tubes. An additional coating of PDMS was applied. After curing, the patterned PDMS was cut with a scalpel and lifted off the mold. The Teflon tubing was removed with forceps. The pattern was replicated with high fidelity as shown in Figure 1(b). The patterned PDMS was bonded to a flat PDMS sheet with an oxygen plasma (Technics, Inc., Dublin, CA). Female Luer fittings (1/8 inch, Cole-Parmer) were connected to the inlet and outlet. Prior to cell seeding, the devices were sterilized by autoclaving. A macroscopic view of the device is shown in Figure 2.



(a)



(b)

Fig. 1. *(a) Scanning electron micrograph of the vascular network on a silicon wafer. A lithographic process (image reversal) has been applied to produce raised channels against an etched background. (b) Scanning electron micrograph of the vascular network in PDMS. The pattern has been transferred with high fidelity to PDMS by replica molding.*

Cell culture

An immortalized human microvascular endothelial cell line (HMEC-1) that retains the morphologic, phenotypic and functional characteristics of normal human microvascular endothelial cells was used in this study (Ades et al., 1992). HMEC-1 cells were cultured in a medium consisting of Dulbeccco's Modified Eagle Medium (Gibco-BRL, Grand Island, NY) supplemented with penicillin (400 U/ml), streptomycin (400 μg/ml), fetal bovine serum (10%) and hydrocortisone acetate (1 μg/ml) (all from

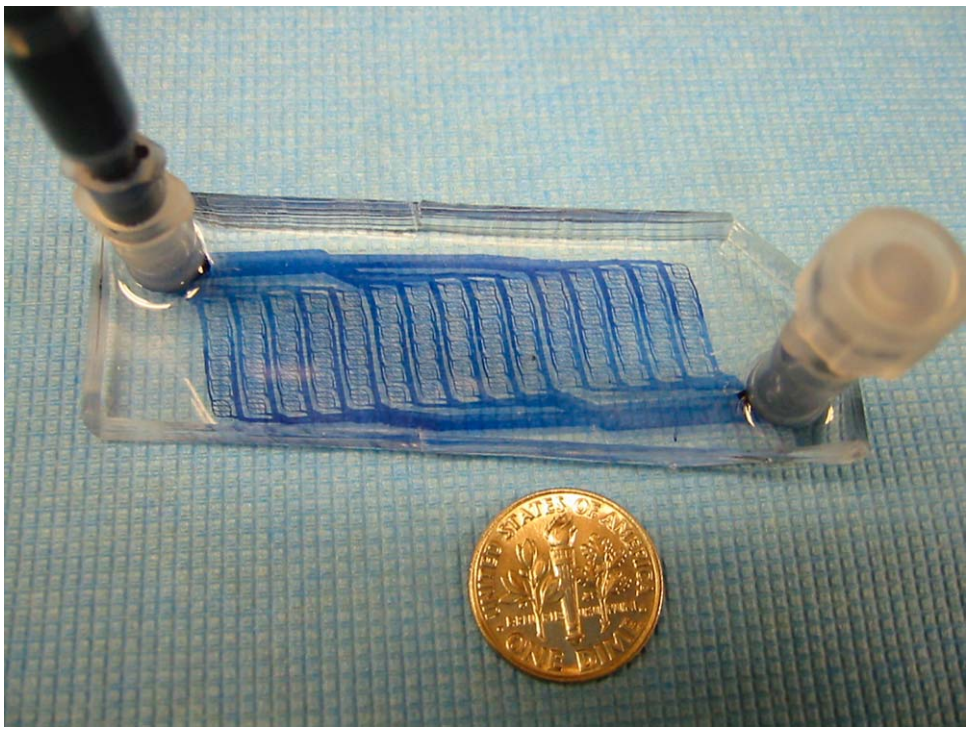


Fig. 2. Macroscopic view of the microfabricated PDMS network. The patterned PDMS has been bonded to a flat PDMS sheet to obtain closed channels. The channels have been filled with ink for visualization. The network has a surface area of 13.69 cm^2 and an internal volume of 21.3 mm^3. The width of the capillaries ranges from 5 mm (main channels near inlet and outlet) to 35 μm (capillary bed in the center). All channels have a depth of 35 μm.

Sigma, St. Louis, MO). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Prior to cell seeding, the channels were coated with a purified collagen solution (Cohesion, Palo Alto, CA) and incubated overnight to facilitate cell adhesion. Confluent HMEC-1 cells were detached with trypsin, resuspended in culture medium and added to the vascular networks at a concentration of 20 million cells/ml. After 3 hours of static incubation, the devices were turned upside down to let the cells attach to the ceiling of the channels. After 6 hours of static incubation, the inlet was connected to a medium-containing syringe, a custom-made oxygenator and air trap. The outlet was connected to silastic tubing leading to a waste container for the effluent. Culture medium was perfused through the channels at a flow rate of 0.5 ml/hr using a syringe pump (Harvard Apparatus, Holliston, MA). The entire assembly was contained in an incubator. A schematic representation of the experimental set-up is shown in Figure 3. Cell attachment and viability were monitored daily using a Nikon inverted light microscope.

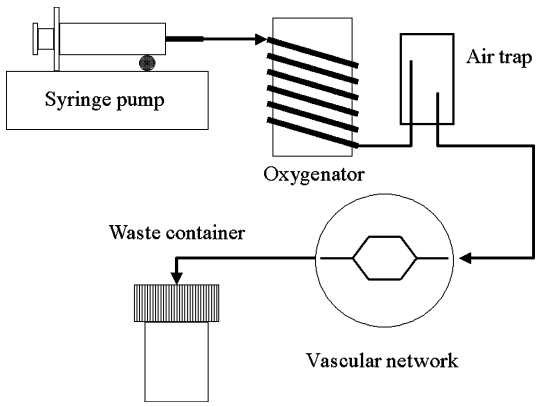


Fig. 3. Schematic representation of the culture system for endothelial cells in PDMS networks. Culture medium is delivered to the cells by a syringe pump through silastic tubing through an oxygenator to enrich the medium with oxygen and an air trap to remove air bubbles. The effluent is collected in a waste container. The entire set-up is contained in an incubator.

Immunohistochemistry

After 14 days of *in vitro* culture, the cells were fixed within the device with 4% paraformaldehyde and double-stained with two endothelial-specific cell markers, CD31 (platelet endothelial cell adhesion molecule, PECAM-1) and von Willebrand Factor (vWF). For CD31 identification, the cells were incubated with the primary antibody, mouse anti-human CD31 (DakoCytomation, Carpinteria, CA). Subsequently, the cells were incubated with green fluorescence-conjugated secondary antibody. For vWF identification, the cells were incubated with the primary

antibody, rabbit anti-human vWF (DakoCytomation) and red fluorescence-conjugated secondary antibody. The cells were analyzed using a Zeiss fluorescence microscope.

Results

Biocompatible microfluidic channels with a vascular topography were made by replica-molding of PDMS from etched silicon wafers. The channels were patent, and there were no leaks between the channels and the connectors as tested by flowing water and dye through the devices using a syringe pump. The devices withstood flow rates of up to 12 ml/min without rupturing or leaking.

Immortalized human microvascular endothelial cells were used in this study. Due to the difficulties associated with the harvest and growth of primary endothelial cells, this cell line offers the benefits of using human cells and rapid expansion. HMEC-1 cells can be passaged up to 50 times and show no sign of senescence. Significantly, HMEC-1 cells retain microvascular endothelial cell characteristics. HMEC-1 cells grow in monolayers and exhibit the typical cobblestone morphology. They express and secrete vWF, and express cell surface molecules typically associated with endothelial cells and cell adhesion molecules.

HMEC-1 cells were cultured in closed PDMS microchannels for 14 days. Figure 4(a) shows the HMEC-1 cells immediately after seeding. The cells are suspended and distributed throughout all channels. Figure 4(b) shows the HMEC-1 cells in the channels after 6 hours of static incubation. The cells have attached to the bottom as indicated by the change in morphology. Culture medium was perfused through the channels at a flow rate of 0.5 ml/hr. The HMEC-1 cells proliferated well within these confined geometries. After one week, the HMEC-1 cells had become confluent and formed a monolayer exhibiting a cobblestone morphology (Figure 4(c)). The culture was continued for up to 2 weeks, and all channels remained covered with a HMEC-1 monolayer (Figure 4(d)). At all times, two focal planes could be observed in light microscopy, indicative of cells attached to the top and bottom of the channels.

The HMEC-1 cells in the microchannels were double-stained with well-established endothelial markers. Figure 5 shows that the HMEC-1 cells express CD31, which is not only an endothelial marker but also a key molecule for monolayer formation. The HMEC-1 cells stain positively for vWF, another important endothelial marker (Figure 6). These results indicate that the HMEC-1 cells form a monolayer and continue to express the normal proteins of endothelial cells in confined geometries.

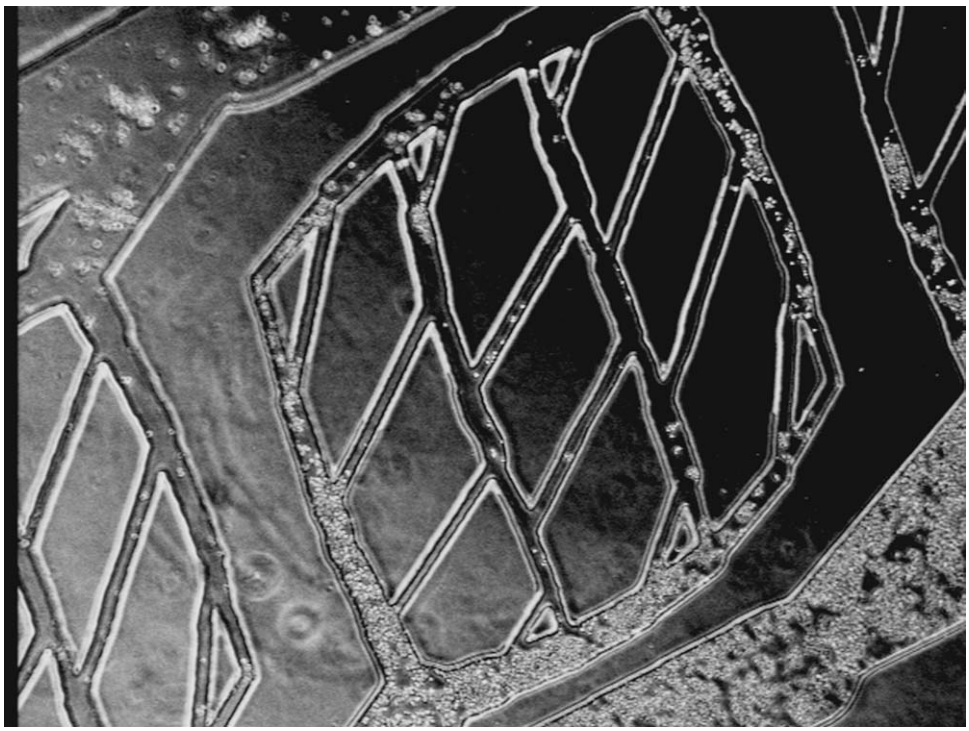
Discussion

Tissue engineering has made significant advances towards creating viable skin, cartilage and bladders (Kojima et al., 2003; Metwalli et al., 2003; Oshima et al., 2003). The ultimate goal of tissue engineering is to alleviate the organ shortage problem by creating large pieces of tissue and whole organs. One of the key challenges is to provide larger structures with sufficient oxygen and nutrition for survival and proliferation. Our approach is to use microfabrication technology to create a vasculature *in vitro*. A process methodology combining silicon microfabrication and polymer replica molding has been developed to form closed channels with a vascular topography in PDMS. HMEC-1 cells were cultured for up to two weeks and maintained their character in these confined geometries.

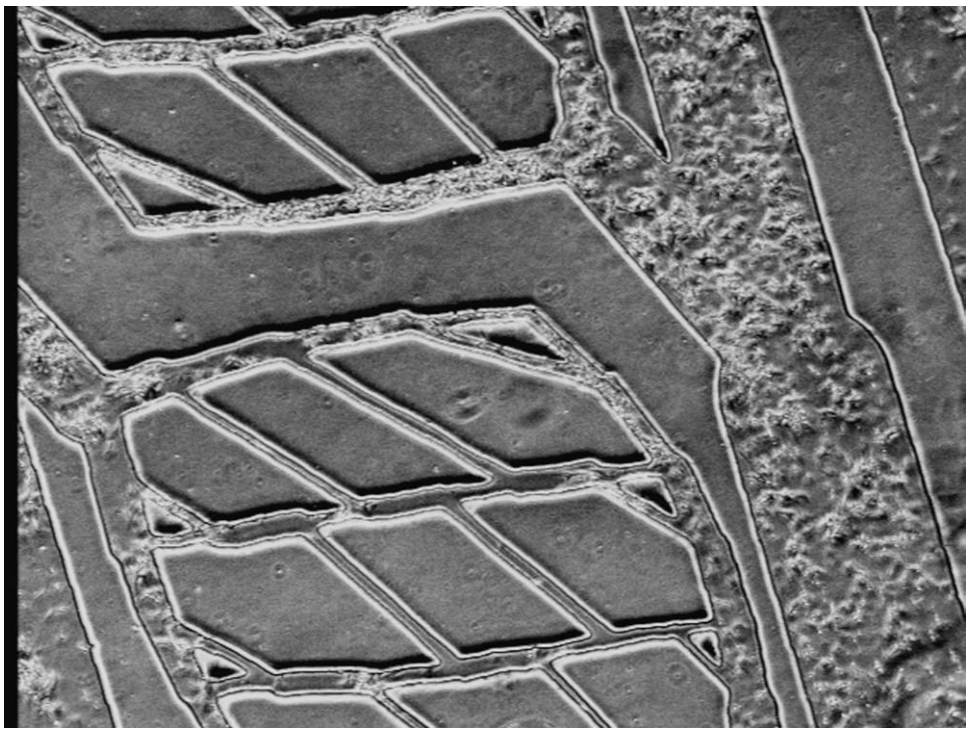
This represents a significant advance since our earlier description of using silicon microfabrication to form sheets of living tissue. In the first approach, cells were cultured on patterned silicon and Pyrex wafers, and lifted off from these 2-D molds (Kaihara et al., 2000). Since then, the process has been advanced in two important areas, i.e., the substrate material and the geometry. The adaptation of the process from rigid and brittle materials, e.g., silicon and glass, to soft and biocompatible materials, e.g., PDMS, has resulted in improved handling and opens up the possibility of *in vivo* studies. In addition, moving from open trenches to closed channels and lining the channels with endothelial cells is an important advance towards engineering a vasculature.

While these results are promising, important questions and challenges remain. First, there is a geometrical difference between native vessels and the microfabricated network. Although the trenches in the silicon wafer have rounded bottoms, the microfabricated channels have rectangular lumen, compared to the round lumen in native vessels. Current experiments are underway to assess the effects of the lumen shape with regards to flow, pressure changes and turbulence and compare them to the predictions of our computational model. The process methodology can be easily adapted to create channels with round lumen by bonding two patterned PDMS templates with semicircular trenches.

The observation of two focal planes in light microscopy and the seeding protocol indicate that the ceilings and bottoms of the channels are covered with cells. This was also verified by lectin-FITC staining (data not shown). To demonstrate that the sidewalls were also lined with cells, confocal microscopy and SEM were attempted. Due to the overall thickness of the device, confocal microscopy did not yield images of sufficient quality. Preparation of freeze-fractured samples for cross-sectional visualization by SEM was also unsuccessful as the endothelial cells



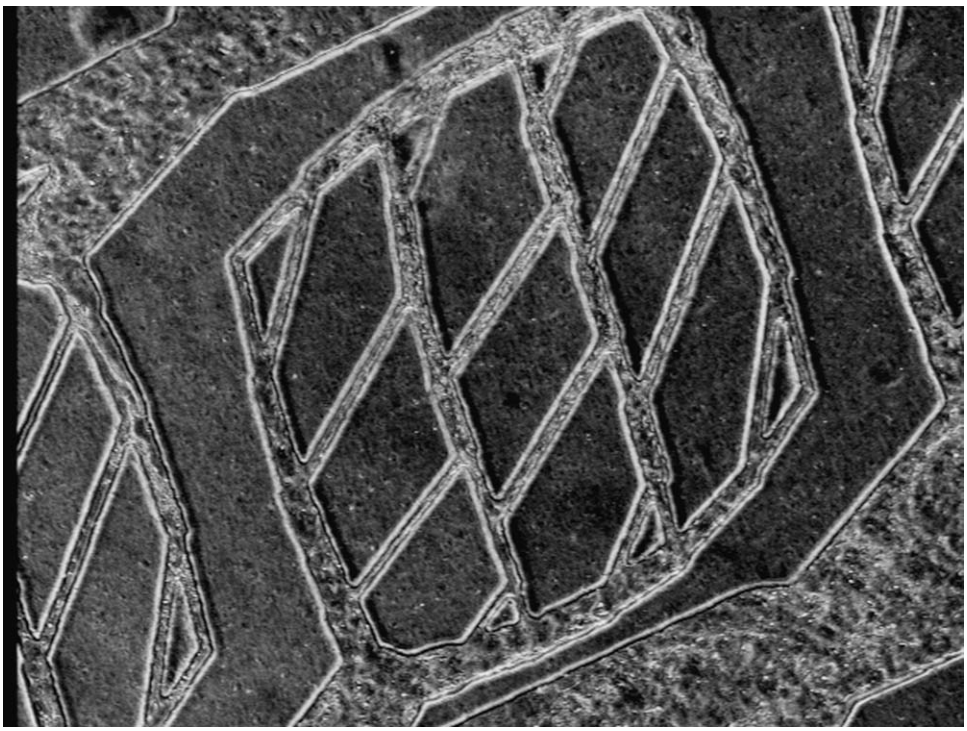
(a)



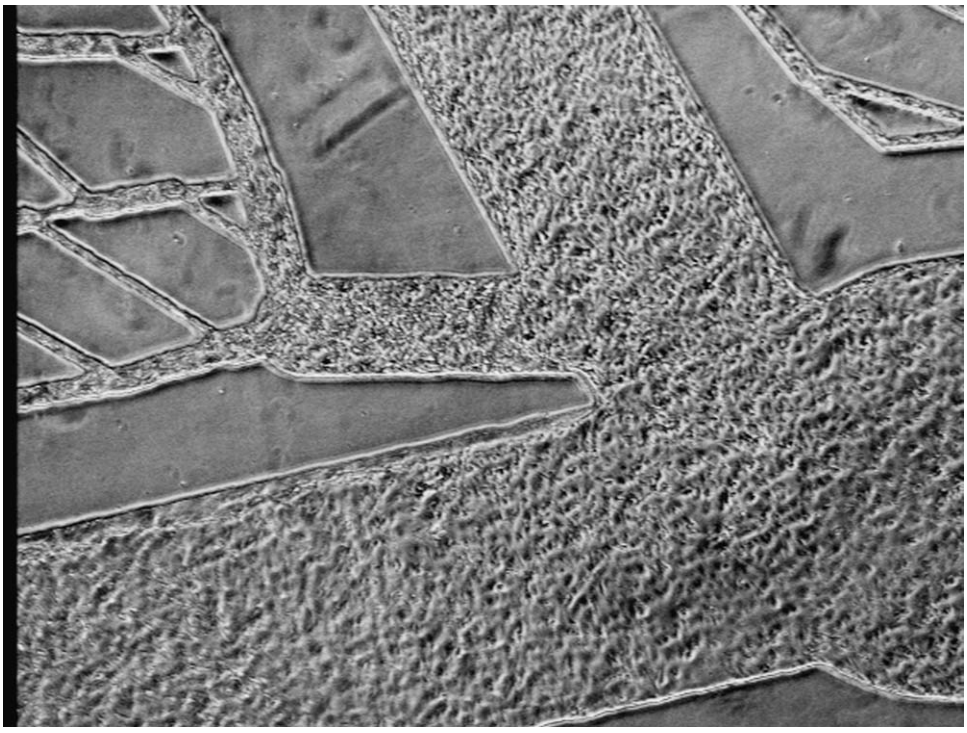
(b)

Fig. 4. *(a) HMEC-1 cells immediately after seeding in the networks. Original magnification 50×. (b) HMEC-1 cells have attached to collagen-coated PDMS surfaces after 6 hours. Original magnification 50×. (c) HMEC-1 cells form a confluent monolayer in the networks around 7 days. Original magnification 50×. (d) HMEC-1 cells were maintained in culture for 14 days. Original magnification 50×.*

(Continued on next page.)

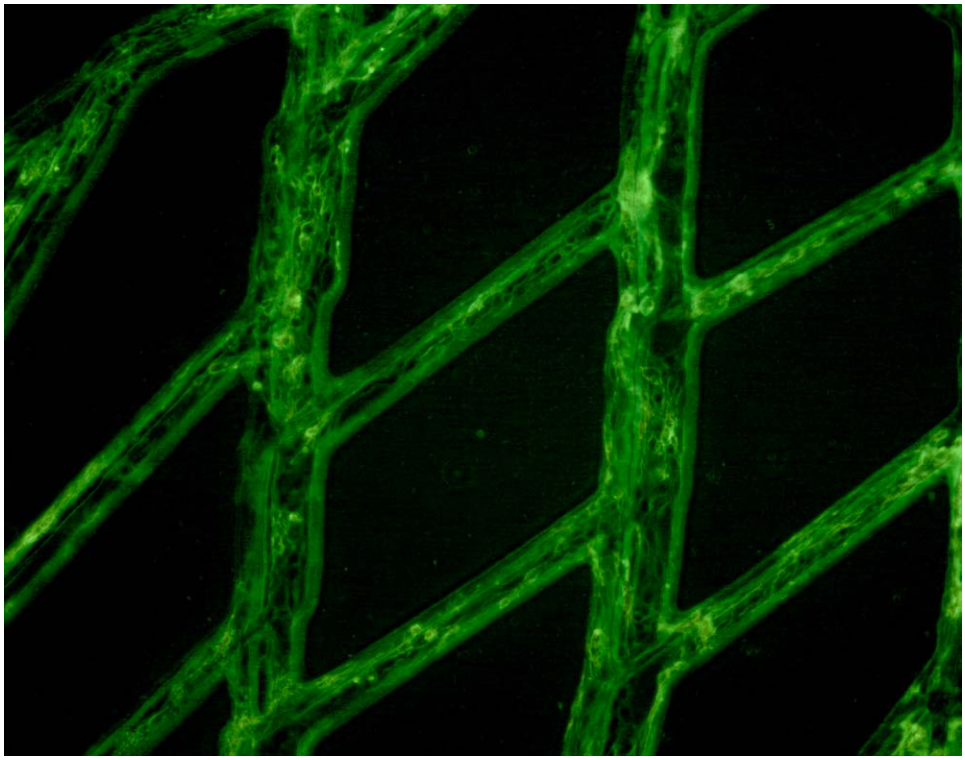


(c)

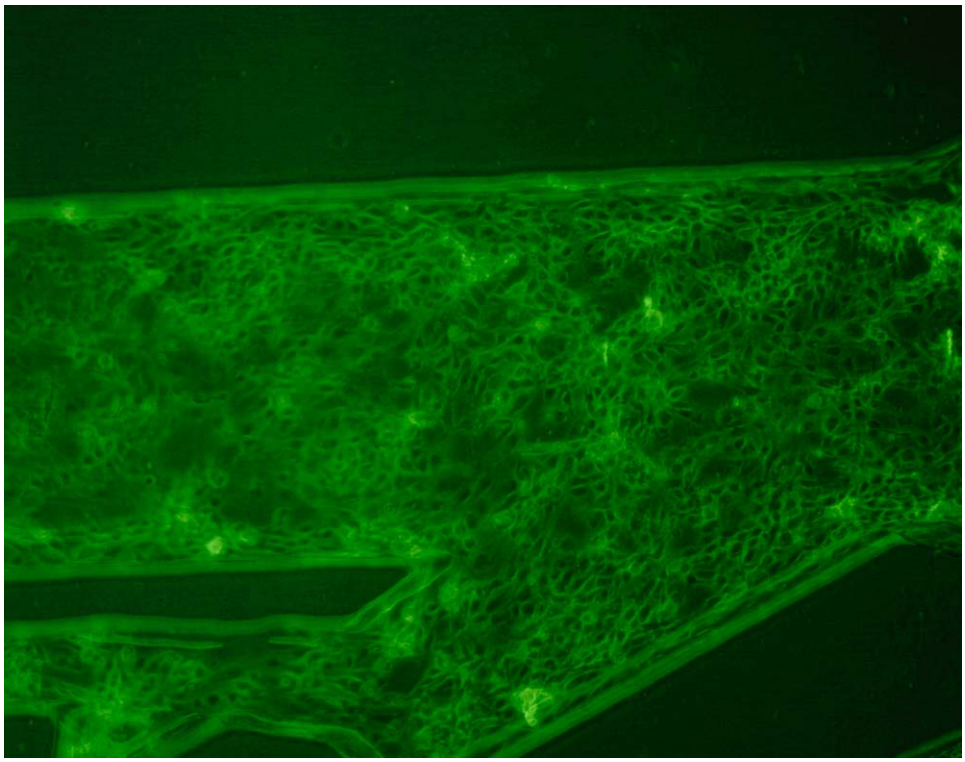


(d)

Fig. 4. *(Continued.)*

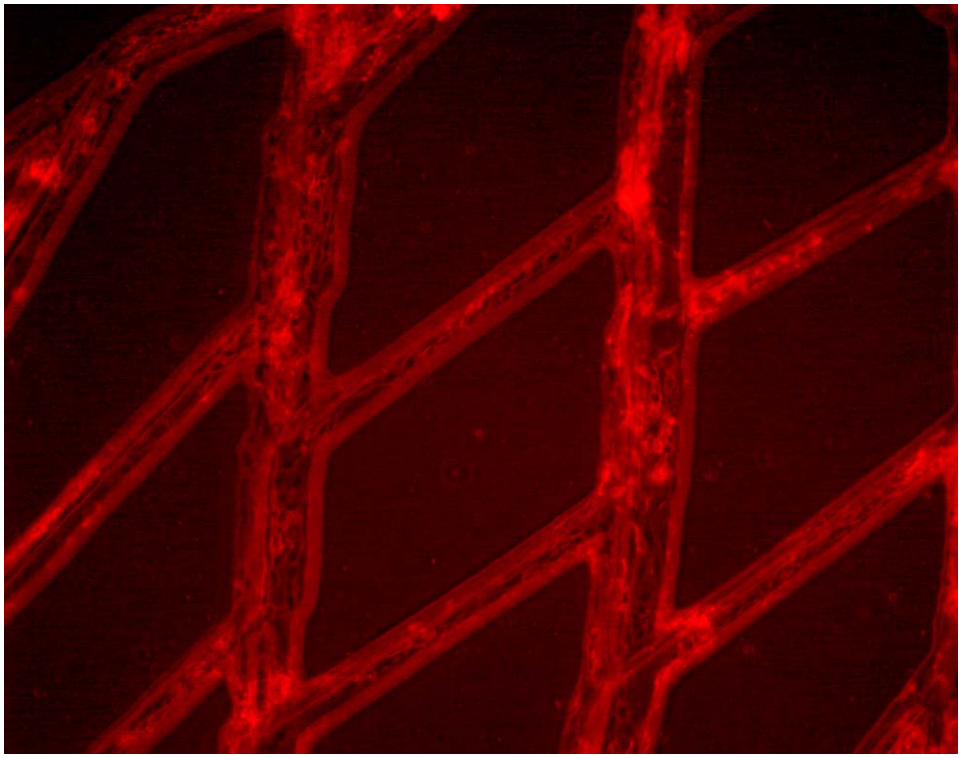


(a)

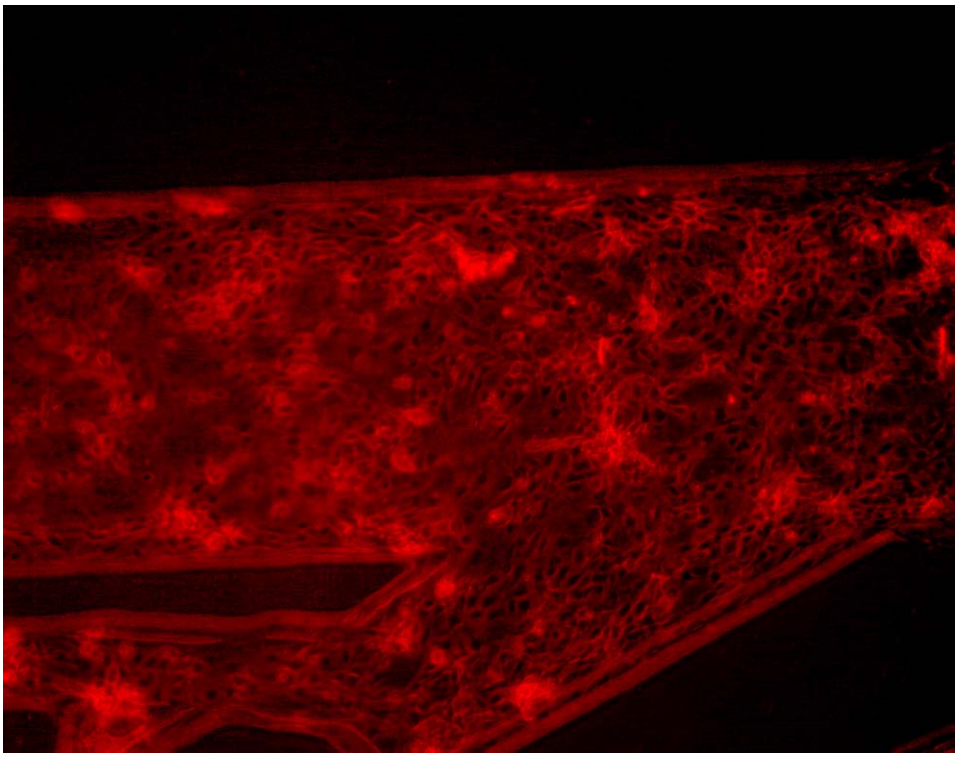


(b)

Fig. 5. *CD31 staining of HMEC-1 cells lining the channels in (a) capillaries (original magnification 200×); and (b) peripheral channels (original magnification 100×). CD31 is an endothelial-specific marker and a key molecule for single layer formation.*



(a)



(b)

Fig. 6. *vWF staining of HMEC-1 cells lining the channels in (a) capillaries (original magnification 200×); and (b) peripheral channels (original magnification 100×), at the same location as Figure 5. vWF is widely used as a phenotype marker for endothelial cells.*

were damaged and lifted off the PDMS. Current efforts are devoted to creating thinner devices for direct visualization using confocal microscopy and improving SEM specimen preparation.

Although PDMS is an inert and biocompatible material, blood clotting eventually occurs. To reduce thrombogenicity and avoid coagulation-related problems, it is appealing to coat surfaces that are in contact with blood with endothelial cells. For such endothelialized devices to be clinically useful, it is imperative to demonstrate long-term viability and function, especially in the presence of physiological flow rates. While short-term viability and expression of endothelial-specific markers have been demonstrated, further characterization of the endothelium is required. Since PDMS is a dense material, the barrier properties of endothelial cells cannot be assessed. However, other vital functions, e.g., endothelial cell nitric oxide synthase (ecNOS) and its response to fluid shear stress and other hemodynamic stimuli, e.g., pulsatile flow, will be assessed in the current system.

In conclusion, an integrated approach combining computational analysis, silicon microfabrication, polymer processing and cell biology has been developed to create endothelialized networks with a vascular geometry. The results demonstrate the feasibility of the concept in a 2-D biocompatible system. Additional research efforts are devoted to design and materials. In order to create an *in vitro* organ analogue, the vasculature must be integrated with the parenchyma. To this end, dual-compartment devices have been recently developed in which the vascular and parenchymal compartments are separated by a semipermeable membrane. The next stage will be the creation of 3-D structures, which requires additional advances in design and polymer processing since silicon microfabrication is an inherently planar or 2-D process. Finally, the processing methodology will be transferred to biodegradable materials to allow for *in vivo* applications.

Acknowledgments

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