

Endothelialized Microvasculature Based on a Biodegradable Elastomer

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ABSTRACT

Vital organs maintain dense microvasculature to sustain the proper function of their cells. For tissue-engineered organs to function properly, artificial capillary networks must be developed. We have microfabricated capillary networks with a biodegradable and biocompatible elastomer, poly(glycerol sebacate) (PGS). We etched capillary patterns onto silicon wafers by standard microelectromechanical systems (MEMS) techniques. The resultant silicon wafers served as micromolds for the devices. We bond the patterned PGS film with a flat film to create capillary networks that were perfused with a syringe pump at a physiological flow rate. The devices were endothelialized under flow conditions, and part of the lumens reached confluence within 14 days of culture. This approach may lead to tissue-engineered microvasculature that is critical in vital organs engineering.

INTRODUCTION

EACH YEAR in the United States alone, millions of people suffer from end-stage organ failure and tissue loss, resulting in more than \$400 billion in health care costs.¹ Although 10% of these patients benefit from organ transplantation, the majority of patients perish while waiting for donor organs.¹ To address this problem, the field of tissue engineering emerged with the promise of eventually solving the organ donor shortage. Although tissue engineering is a relatively young field, growing living cells on three-dimensional scaffolds to form whole tissues capable of normal biological functions has become an active area of research.^{2,3}

To date, the most successful tissue-engineering applications are skin and cartilage, which have relatively low requirements for nutrients and oxygen and can be supported by the host vasculature. Development of an artificial microvasculature is critical to move tissue-engineered vital organs into the clinics to benefit patients with end-stage organ failure. Without proper vascularization, tissue-engineered organs rely mainly on host vasculature for oxygen, nutrients, and waste removal.^{2,3} As a result, the complexity and the thickness of these tissues are limited by the mass transfer properties of the scaffold material. Without an intrinsic capillary network, the maximal thickness of engineered tissue is approximately 150–200 μm because of oxygen diffusion limitations.^{4,5}

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Vital organs, such as heart, liver, and kidney, have high metabolic rates, which further limit the maximal thickness of engineered tissue. This problem can be overcome by building capillary networks within the engineered tissue to transport oxygen and nutrients to remove waste.⁶

We have developed a novel biodegradable and biocompatible elastomer, poly(glycerol sebacate) (PGS) that matches the mechanical properties of veins.⁷ In addition, we have shown that PGS causes no chronic inflammation when implanted subcutaneously in animal models. Different types of cells, including endothelial cells, adhere to and proliferate on unmodified PGS surfaces. Here, we describe the engineering of PGS capillary networks with part of the lumens covered by endothelial cells to near confluence as judged by microscopic observation. These results suggest the potential of PGS in creating tissue-engineered vital organs.

MATERIALS AND METHODS

Microfabrication of silicon wafers

Standard 4-in. silicon wafers were processed by standard lithography techniques as described earlier.⁸ A negative mold of the designed capillary pattern was fabricated such that the background was etched away, leaving the raised channels of the capillary networks. The silicon wafers were cleaned in a solution containing 3 parts H_2SO_4 (100%) and 1 part H_2O_2 (30%) at 140°C for 20 min, rinsed with deionized water, and dried in nitrogen gas (PSC-101; Semitool, Kalispell, MT). Last, the wafers were treated with oxygen plasma for 1 min (MARCH Px-250-03/01, 200 mTorr, 80 W).

Each wafer was spin-coated with 3 mL of a $0.2\text{-}\mu\text{m}$ pore size filtered 90% sucrose solution at 1000 rpm for 30 s (PWM32-PS-CB15; Headway Research, Garland, TX). The sucrose layer was dried by heating the wafers on a 65°C hotplate for 1 min. The thickness of the sucrose layer was measured by ellipsometry, using the Filmetrics (San Diego, CA) F20 spectrometry system. The sucrose layer was further dried by baking the coated wafers at 120°C under vacuum (5 mTorr) for 24 h.

Fabrication of PGS capillary networks

The capillary network pattern was transferred from the silicon micromold to PGS according to procedures illustrated in Fig. 1a. For a PGS film 1 mm in thickness, 6.8 ± 0.1 g of prepolymer⁷ was melted and was poured over the silicon wafer. The wafer was placed on a hotplate at 170°C until the prepolymer formed a uniform layer over the whole wafer. The prepolymer was cured at 150°C under vacuum (5 mTorr) for 13 h.

Before releasing the PGS layer, incisions were made in the PGS to demarcate the final size of the patterned pieces. The silicon micromold was then soaked in deionized water for 24 h. Subsequently, the PGS layer could be easily removed from the wafer. The patterned PGS films were then soaked in 70% ethanol for 24 h to remove any residual oligomers or unreacted starting materials.

A flat PGS layer was polymerized on a 38×75 mm glass slide. Before use, the glass slide was cleaned with 70% ethanol. PGS prepolymer (1.8 ± 0.1 g) was melted on each glass slide on a 170°C hotplate until the prepolymer formed a uniform layer. The prepolymer was cured at 120°C under vacuum (5 mTorr) for 28 h. The

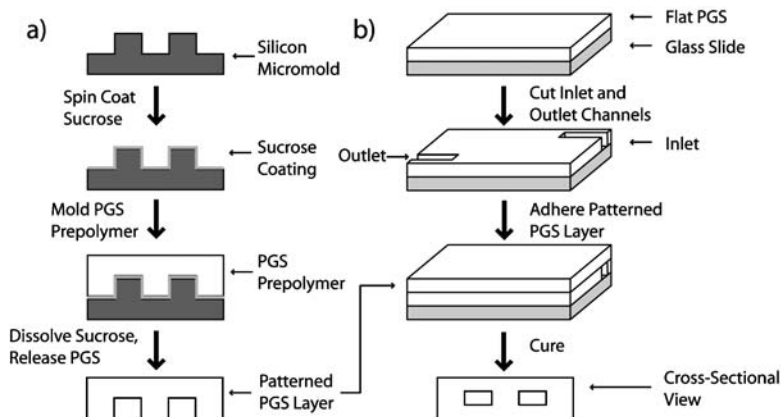


FIG. 1. (a) Schematic representation of the molding process: each silicon wafer is spin coated with a 90% sucrose solution. The PGS prepolymer is poured over the coated wafer and cured. The patterned PGS film is removed after the dissolution of the sucrose layer. (b) Schematic representation of the bonding process: PGS prepolymer is polymerized over a glass slide. Notches are cut into the PGS at the inlet and outlet positions. The molded PGS layer is placed patterned side down onto the flat layer. Further curing of PGS facilitates bonding between the two layers. Scheme not drawn to scale.

flat PGS films were removed from the oven and were allowed to cool to room temperature. Using the silicon micromold as a guide, inlet and outlet channels measuring 20×2 mm were cut out on the opposite ends of the flat film (Fig. 1b). The inlet and outlet channels in the patterned PGS film were aligned with the corresponding channels in the PGS flat film, and the two layers were allowed to adhere to each other, which occurred readily. The whole device was further cured at 120°C under vacuum (5 mTorr) for 24 h to permanently bond the two layers (Fig. 1b). Glass petri dishes were also sterilized in the oven during this final polymerization step. The finished devices were immediately transferred to the sterilized petri dish on opening of the vacuum oven. The devices were kept in the sterile petri dish until final assembly in a sterile lamellar flow hood.

To create ports for attaching the devices to a perfusion system, sterile 23- and 20-gauge blunt-end needles were inserted into the inlet and outlet channels, respectively. Empirically, the larger outlet needle seems to reduce resistance in the outlet. These needles were set in place with polyurethane glue. The finished device was approximately $38 \times 75 \times 1$ mm. The strength of the bond was assessed by perfusing the network devices with phosphate-buffered saline (PBS; Sigma, St. Louis, MO) at varying flow rates up to approximately 50 mL/h. The patency of the channels and the quality of the bond between PGS layers were assessed by fluorescence microscopy and scanning electron microscopy (SEM). Briefly, rhodamine B aqueous solution (0.01 mM) was manually injected into the finished devices through the inlet. A fluorescence microscopy image (Axiovert 200 fluorescence microscope; Zeiss, Thornwood, NY) was taken immediately after the whole device was filled with the dye. The samples for SEM were obtained by cutting the finished devices cross-sectionally and by mounting pieces (approximately 3 mm high) on aluminum SEM studs. The samples were observed with a Philips FEI XL-30 FEG environmental scanning electron microscope (FEI, Hillsboro, OR) under 2 Torr, 10 kV beam, 2.0 spot size, with a gas-phase secondary electron detector.

Endothelialization

The devices were seeded with primary human umbilical vein endothelial cells (HUVECs) obtained from Cambrex Bio Science Walkersville, Walkersville, MD). These cells were cultured in EBM-2 base medium (Cambrex Bio Science Walkersville) supplemented with an EGM-2 SingleQuot kit (Cambrex Bio Science Walkersville). The passage number for the HUVECs used for seeding devices ranged from passage 3 to passage 8. The harvested cells were centrifuged at 1500 rpm ($380 \times g$) for 5 min and resuspended in EBM-2 culture medium to yield a concentration of approximately 1×10^7 cells/mL.

Each device was seeded with 1 mL of this single-cell suspension. The newly seeded devices were placed in a 5% CO_2 incubator at 37°C and 100% humidity. The cells were observed periodically during this static culture phase to assess when the cells were no longer floating within the channels and when they appeared to adhere to the lumens. The cells remained under these static conditions for up to 1 h, at which point most of the cells adhered to the lumen of the channels. The device was attached to a perfusion system, located within the 5% CO_2 incubator at 37°C and 100% relative humidity. A six-port syringe pump (model NE-1600 Multi-Phaser; New Era Pump Systems, Farmingdale, NY) perfused EGM-2 culture medium through the devices at a rate of 100 $\mu\text{L}/\text{h}$. The temperature and CO_2 concentration of the medium was equilibrated while it passed through a 1-m-long silicone tubing loop inside the incubator. The used medium was collected in a sterile glass vial fitted with a 0.2- μm syringe filter after the medium had passed through the device. The devices remained under the perfusion culture conditions for 4 weeks. The endothelialized capillary networks were visualized daily with a Zeiss Axiovert 200 microscope at $\times 2.5$, $\times 5$, $\times 10$, and $\times 40$ power. Digital images were obtained with AxioVision 3.1 software.

In addition, six devices were coated before cell seeding with peptides that facilitate cell adhesion. Approximately 1 mL of a 5- $\mu\text{g}/\text{mL}$ solution of fibronectin active fragment GRGDS (Peptide Institute, Osaka, Japan) in sterile PBS was manually injected into a network device. The device was then incubated at 25°C for 30 min before cell seeding. These devices were seeded with HUVECs and cultured as described above.

RESULTS

Coating of silicon micromolds

Silicon micromolds were fabricated by standard soft photolithography techniques as previously described,⁸ except that these silicon wafers do not undergo fluoropolymer pacification during the last step of the microfabrication process; once exposed to oxygen in the air, silicon atoms on the surfaces would be oxidized to SiO_2 molecules, which would render the surfaces hydrophilic. To facilitate the subsequent release of patterned PGS from the silicon wafers, we coated them with a sacrificial layer of sucrose (Fig. 1). The silicon micromolds were treated with oxygen plasma to remove any residual contaminants so that the sucrose coating was uniform across the whole wafer. For the 42 coated wafers that were measured by ellipsometry, the mean thickness of the sucrose coating was 3.5 μm with a standard deviation of 0.2 μm . The sucrose coating did not crystallize on the wafer at macroscopic level for periods up to 1 year after the spin-coating process.

Molding PGS capillary networks

We spread molten PGS prepolymer evenly on the sucrose-coated silicon micromolds. Polymerization of PGS at 150°C and 5 mTorr overnight yielded a film firm enough to maintain the integrity of the capillary channels. The PGS film released readily from the silicon wafer once the sucrose coating was dissolved. No distortion of the channels was observed when viewed microscopically (Fig. 2).

To promote cell growth and to reduce acidity within the capillary network device, residual unreacted monomers and oligomers were removed from the patterned PGS film by washing with ethanol before further processing. This wash caused an initial swelling of the PGS film that was reversible on desiccation. The majority of the ethanol was removed on desiccation for 24 h; the residual volatiles were removed in the subsequent bonding process (Fig. 1b). The resultant patterned PGS layers show features nearly identical to those of the silicon micromold (Fig. 2). Visually, they appeared as transparent

and flexible thin films with embedded capillary channels (Fig. 3a).

We enclosed the open capillary channels in the patterned PGS layer by bonding it with a flat PGS film. The flat film was lightly cured and sticky to enhance its bonding with the patterned layer. Initially, the bonding process had led to frequent adhesion and occlusion between the top and bottom of the inlet and the outlet (the widest channels, 3000 μm wide and 30 μm high). Most likely, because the width-to-height aspect ratio of the inlet and outlet channels is so large, a slight relaxation of the elastomer was enough to allow the top of the channel to touch and adhere to the bottom. We overcame this problem by cutting inlet and outlet ports into the flat film to decrease the width-to-height aspect ratio. This step further facilitated the insertion of needles into the inlet and the outlet channels through the side of the device. The final dimensions of the whole devices were approximately $38 \times 75 \times 1$ mm (Fig. 3b). The channel width varied from 3 mm at the inlet and outlet to 45 μm at the finest capillaries. The depth of the channels was constant throughout the device at 30 μm .

Because PGS is transparent, the patency and the integrity of even the smallest capillaries could be observed by optical microscopy. The bonding (second curing) of the combined PGS layers yielded a strong bond between the two films, such that the capillary network could withstand pressures generated by flow rates up to approximately 150 and 1000 cm^3/s within the largest and smallest channels, respectively. Even after 4 weeks in a 5% CO_2 incubator at 37°C and 100% relative humidity, this bond can still withstand pressures generated by physiologic flow (0.05 cm^3/s).

The cross-section of the capillary channels was trapezoidal (Fig. 4a), most likely because the sucrose coating solution preferentially accumulated at the corners of the silicon micromold because of surface tension. The patency of the capillary network was also demonstrated by fluorescence microscopy (Fig. 4b). The image was taken after the capillary networks were filled with an aqueous rhodamine B solution. No leakage or occlusion was observed throughout the network.

Endothelialization

More than 50 devices were seeded with HUVECs, approximately 30 of which were used to optimize the cell-seeding and endothelialization procedure in terms of the initial cell-seeding density, the perfusion rate, and the modification with cell adhesion-promoting peptides. We endothelialized 24 devices under optimized conditions as described in Materials and Methods, 6 of which were modified with GRGDS peptides. While the HUVECs were adherent to the native PGS, microscopic observations suggested that GRGDS pentapeptide promoted a

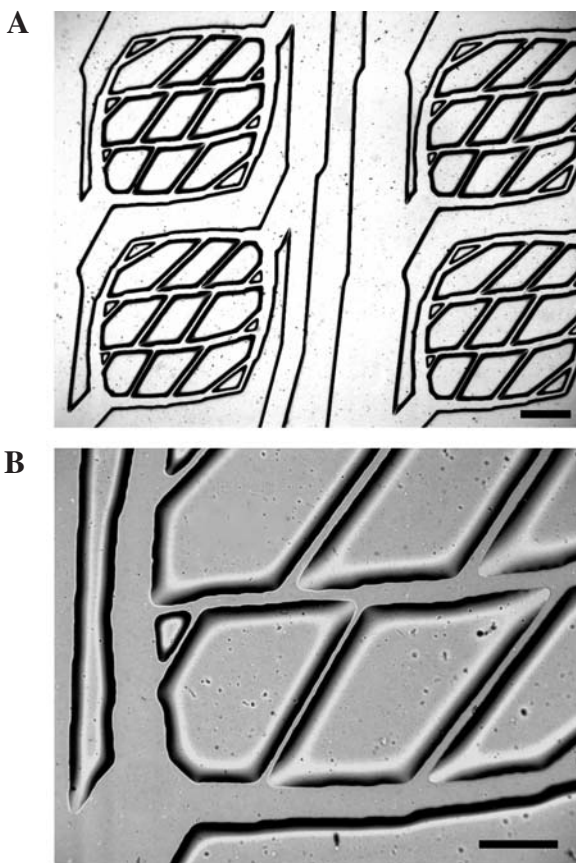


FIG. 2. Photomicrographs of patterned PGS. PGS film with the imprinted capillary network pattern is shown. Original magnifications: (a) $\times 2.5$; (b) $\times 10$. Scale bars: (a) 500 μm ; (b) 200 μm .

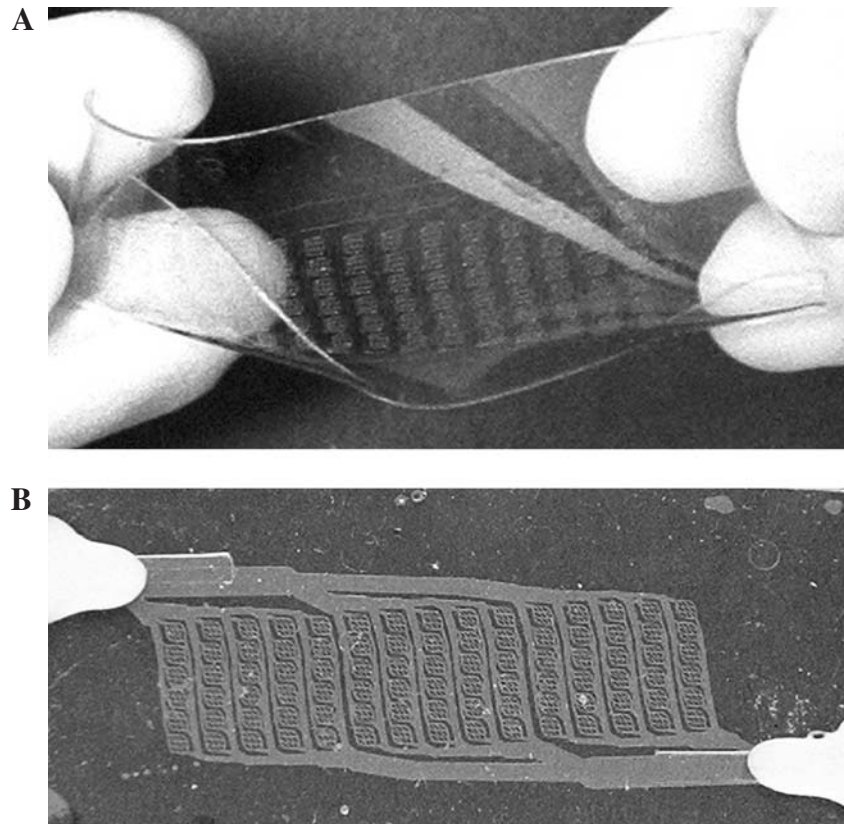


FIG. 3. (a) The patterned elastomeric PGS film forms the top layer of the capillary network. (b) The final device with polyurethane-sealed connections to an external circulation.

more uniform distribution and adhesion of cells within the device.

Observation of the HUVECs immediately after seeding the capillaries demonstrated that the rounded cells were floating within the medium and were moving ran-

domly. Within 1 h under static culture conditions, the cells still appeared rounded but adherent to the lumen of the capillaries because they were no longer moving in the channels when the devices were tilted back and forth. Perfusion was started once the majority of the cells ap-

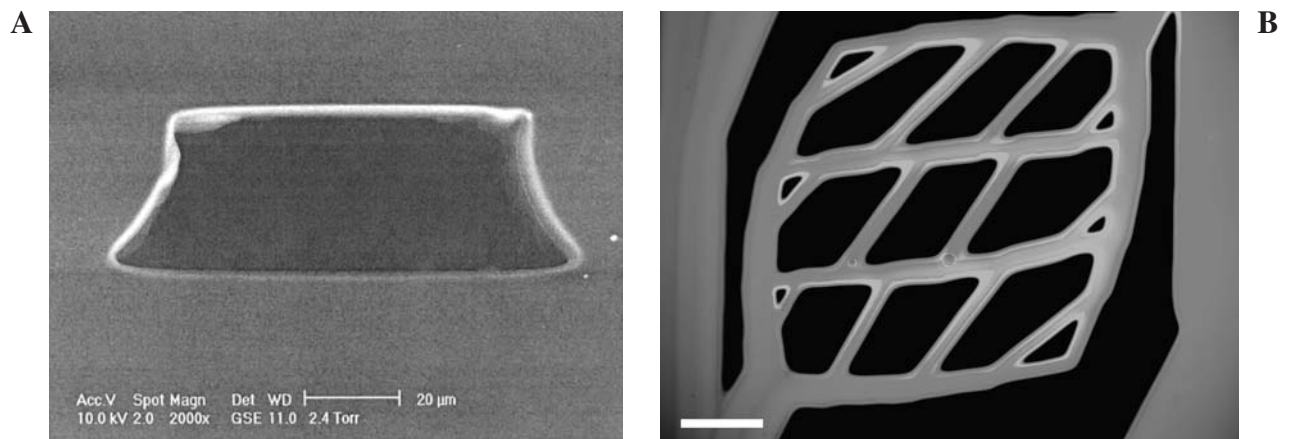


FIG. 4. (a) Cross-sectional view of a bonded capillary after curing (SEM; original magnification, $\times 2000$) confirms that the channels are sealed without leakage or occlusion. (b) Fluorescence microscopy (rhodamine B; original magnification, $\times 5$) also demonstrates the patency of the bonded capillary networks. The two bubble-like features close to the center are artifacts. Scale bar: $500 \mu\text{m}$.

peared adherent. After the first 24 h of perfusion, the non-adherent cells had been flushed from the device, and the remaining cells had a flattened appearance, which suggests that they were adherent to the device. During the first 10 days of culture, the cells proliferated as indicated by an increase in number and density of the cells within the device. The cell expansion slowed after 10 days and parts of the capillary networks were covered by nearly confluent endothelial cells within 14 days (Fig. 5). The cells remained stable under these culture conditions for at least 4 weeks.

DISCUSSION

This study demonstrates that PGS is a potential candidate for a tissue-engineered microvascular network scaffold. PGS has been imprinted with a capillary network pattern using microfabricated silicon wafers as

molds. Furthermore, a fully enclosed capillary network in PGS has been fabricated, perfused, and endothelialized. To ensure optimal blood flow and mass transport characteristics, these microvascular networks have been designed on the basis of computational models that take into account the particulate nature and complex multi-phase rheology of blood, characteristics that are important in microcirculation.⁹

A few attempts have been made to create three-dimensional microvascular networks. A tissue-engineered hepatic tissue containing vascularized sinusoids was created by a three-dimensional printing process.^{10,11} The polymeric scaffold presented features of vessels and parenchymal space. The feature resolution was approximately 200 μm . Although this technique was useful for engineering hepatic sinusoids, it would be difficult to fabricate capillaries with diameters on the order of 5 μm .

Microelectromechanical systems (MEMS) technology has been successfully applied to etch network patterns,

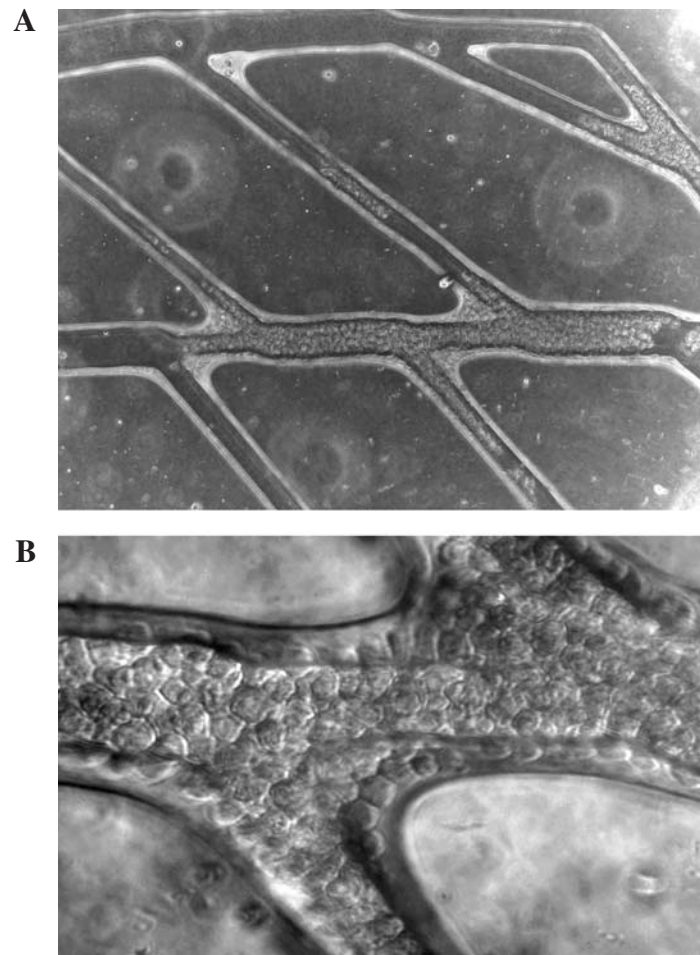


FIG. 5. Photomicrographs of endothelialized capillary network: Endothelial cells reached confluence at various portions of the PGS capillaries within 14 days. Original magnification: (a) $\times 10$; (b) $\times 40$. The large bubble-like features in (a) are artifacts.

of which the smallest channels are 10 μm , into silicon and Pyrex surfaces.¹² Endothelial cells were able to grow within the channels, and these two-dimensional cell layers were lifted from the surface. Although these capillaries can be incorporated into functional devices, this method is limited to generating only monolayer capillary networks.

To create three-dimensional capillary networks, soft lithography techniques were applied to mold polydimethylsiloxane (PDMS), using the network patterns etched into silicon wafers.⁸ These patterned layers of PDMS were irreversibly bonded to flat PDMS layers to create enclosed network channels that were seeded with endothelial cells that grew to near confluence. Other groups have also demonstrated the ease of fabricating PDMS microfluidic devices.^{13,14} These devices, however, might not be suitable for tissue-engineering organs, because PDMS is not biodegradable.

Another study used poly(lactide-co-glycolide) (PLG), a biodegradable polymer, to build artificial capillary networks.¹⁵ When coated with fibronectin, endothelial cells grew to near confluence within the enclosed networks. PLG is used widely in biomedical engineering; however, it is rigid and swells significantly when implanted *in vivo*.¹⁶ The biocompatibility of PLG may not be satisfactory because it can induce chronic inflammation *in vivo*.⁷ Scaffolding materials having mechanical properties that better match the characteristics of the native blood vessels may be advantageous for tissue-engineering capillary networks.

In comparison with the network devices made with PDMS and PLG, endothelial cells can adhere to PGS network devices without the use of adhesion proteins. The addition of fibronectin tends to improve endothelial cell adhesion and proliferation within the enclosed PGS network. Because these cells were maintained in culture for periods up to 4 weeks, this approach seems to hold promise for tissue-engineering vascularized organs.

We have demonstrated the micropatterning and endothelialization of PGS-containing capillary networks with channels of different widths but of the same depth. Natural blood vessels narrow in all three dimensions when transitioning from arterioles and venules to capillaries. To achieve a more physiologic flow pattern, we are currently investigating molding PGS network patterns with micromolds of varying depths such that with each bifurcation, the dimension of the channels decreases. In addition, the cross-section of these channels is more cylindrical. Capillary networks fabricated from these molds would better mimic natural vasculature and may provide more physiological flow and mass transport patterns. We are also attempting to build multiple layers of the artificial vasculature stacked with interdigitating layers of mesenchymal cells. The tissue-engineered mi-

crovasculature would provide the necessary oxygen, nutrients, and waste removal for the mesenchymal cells. The resultant constructs could potentially lead to vascularized tissue-engineered vital organs.

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