Microvessel manifold for perfusion and media exchange in three-dimensional cell cultures

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Integrating a perfusable microvasculature system *in vitro* is a substantial challenge for “on-chip” tissue models. We have developed an inclusive on-chip platform that is capable of maintaining laminar flow through porous biosynthetic microvessels. The biomimetic microfluidic device is able to deliver and generate a steady perfusion of media containing small-molecule nutrients, drugs, and gases in three-dimensional cell cultures, while replicating flow-induced mechanical stimuli. Here, we characterize the diffusion of small molecules from the perfusate, across the microvessel wall, and into the matrix of a 3D cell culture. [<http://dx.doi.org/10.1063/1.4963145>]

INTRODUCTION

A major limitation of *in vitro* tissue engineering is the inability to adequately transport materials into and out of large dense tissue constructs ($>1\text{ cm}^3$).^{1,2} Nutrient and waste diffusion throughout the three-dimensional (3D) support matrix significantly impacts the local microenvironment by altering native protein expression,^{3,4} leading to changes in cell viability,⁵ migration,⁶ and proliferation.⁷ To circumvent this limitation and maintain tissue viability, many 3D cell cultures have unrealistically high liquid-to-cell and liquid-to-matrix ratios. Although some perfusion can be achieved by pressure-driven convection throughout the engineered tissue, the synchronistic effect of chemical gradients, circulatory flow, pulsatile stresses, and other physicochemical cues endogenous to native microvasculature are not replicated. Accordingly, 3D cell cultures that are capable of emulating this complex behavior are desirable and necessary for a biomimetic system. Synergistic approaches combining tissue engineering and microfluidics have provided creative strategies to generate such systems.^{8–10}

Many prior vascularization strategies employ templated microchannels throughout 3D cell cultures to mimic or guide the formation of the microvasculature.^{11–17} In some designs, the microchannels represent simple 3D microvascular architectures and provide the ability to control spatiotemporal chemical gradients and mechanical forces.^{18,19} These systems can use acellular or endothelialized microchannels. Recent designs have even elucidated the importance of incorporating additional mural cells to support *in vitro* microvessels. Vascularization can also be achieved by suspending endothelial cells in micro-chambers and introducing vasculogenic factors to initiate the formation of perfusable vessels.^{20,21} Needless to say, the myriad designs to introduce the microvasculature into 3D tissue constructs is both necessary and underpinning many future engineered tissue technologies.

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Of the aforementioned designs, the microdevice template is a prerequisite for all microvessel formation, yet this disregards the emerging development of free-standing engineered blood vessels. These synthetic blood vessels have been constructed from an array of materials, including silicone,^{22,23} cellulose,²⁴ and polyurethane²⁵ and even from human cells.²⁶ These synthetic vessels are typically produced to resemble veins and arteries with typical diameters of greater than 500 μm . Researchers have demonstrated fine-control of the synthetic blood vessels, to reproducibly create the geometries of venules, arterioles, and capillaries.²⁷ We have also reported on a method for fabricating multi-axial microvessels using hydrodynamic shaping methods²⁸ and demonstrated the capability to produce free-standing microvessels containing multiple vascular cell types.²⁹ Very recently, Lewis *et al.* have reported the production of tissues greater than 1 cm in thickness, and their approach relies on the capability to generate *de novo*, perfusable microvasculature which supported the viability of the thick tissue for greater than 6 weeks, thus illustrating the need for perfusable microvasculature to support thick tissue constructs.³⁰ With these technologies, an alternative vascularization strategy to consider is the integration of printed and free-standing microvessels into organ-on-chip or 3D tissue constructs. By utilizing engineered blood vessels, one can reliably and reproducibly control the number and placement of vessels that support thick tissue constructs, a goal that may be especially desirable for quality control in scale manufacturing of organ-on-chip models or human tissues.

Herein, we address the specific technological challenge of integrating free-standing synthetic microvessels into organ-on-a-chip systems. Neither cannulization nor glass micropipette perfusion is applicable for high-throughput development or use with very fine capillaries; therefore, a microfluidic system using custom engineered microvessels was designed, fabricated, and characterized. Our strategy relies on a microfluidic manifold device that organizes an array of synthetic microvessels within a 3D cell culture in such a way that the fluid can be introduced into one end of the microvessels and recovered from the opposite end. The perfusing media will diffuse through the microvessels and supplement the surrounding tissue culture. We demonstrate our strategy by perfusing media through synthetic microvessels integrated within a thick 3D cell culture. This system represents a novel methodology for integrating free-standing synthetic blood vessels into an organ-on-chip, and it can be amenable to use with other synthetic blood vessel technologies.

EXPERIMENTAL

Materials

Poly(ethylene glycol) dimethacrylate (PEGDMA $M_w = 750 \text{ g mol}^{-1}$), poly(ethylene glycol) (PEG-400 $M_w = 400 \text{ g mol}^{-1}$), Irgacure 2959 (I2959), gelatin (Porcine Type A), methacrylic anhydride, and antibiotic-antimycotic solution were purchased from Sigma Aldrich (St. Louis, MO). Dialysis membranes were purchased from SpectrumLabs (Houston, TX). Human neonatal dermal fibroblast, fibroblast basal medium (FBM), serum-free fibroblast growth kit, trypsin-EDTA for primary cells, and phosphate buffered saline (PBS) were purchased from ATCC (Manassas, VA). Calcein-AM, ethidium bromide homodimer, and trypan blue were purchased from Life Technologies (Grand Island, NY). Photo-curable silicone was purchased from Novagard Solutions (Cleveland, OH).

Extracellular matrix synthesis

Gelatin methacrylamide (GelMA) was used as a scaffold for 3D cell culture. We have previously reported the synthesis and pertinent mechanical properties of GelMA.³¹ Briefly, a solution of 10% gelatin in sterile PBS was heated at 60 °C while being stirred until fully dissolved. Methacrylic anhydride was slowly added to a final concentration of 7.5% (v/v). The mixture was allowed to react for 2 h at 60 °C and constant stirring. Following the reaction, the excess methacrylic acid was removed via dialysis for seven days. The pure GelMA was then lyophilized and stored in the dark at 4 °C until used.

Synthetic microvessel fabrication

We have previously described the method for fabricating multi-axial microvessels *via* hydrodynamic shaping and *in situ* photopolymerization of hydrogel macromers.^{28,32} Using the hydrodynamic shaping and *in situ* photopolymerization method, microvessels can be fabricated with inner diameters ranging from 50 to 300 μm and outer diameters ranging from 75 to 500 μm .²⁸ The porosity of PEGDMA microfibers and microvessels is tuned by the weight percent of the PEGDMA macromer solution, and the average diameters of the pores can range from 3 to 30 μm .³² For the reported manifold device, we selected parameters to produce microvessels with inner diameters comparable of arterials and venules. Briefly, a hollow microvessel is formed by microfluidic shaping of three miscible fluid streams, i.e., core, cladding, and sheath fluid. Prior to the fabrication process, the hydroquinone inhibitor was removed from PEGDMA using an inhibitor removal column (SDHR-4, Scientific Polymer Products Inc., Ontario, NY). The cladding fluid was composed of PEDGMA mixed with 0.5 wt. % I2959 in PBS by sonication for 30 min at 60 °C. The sheath and core solutions necessary for microfluidic shaping were composed of matching PEG-400 concentrations in PBS. Syringe pumps were set at 15 $\mu\text{l min}^{-1}$, 30 $\mu\text{l min}^{-1}$, and 60 $\mu\text{l min}^{-1}$ for the core, cladding, and sheath streams, respectively. The fluid streams traversing the microfluidic shaping device were exposed to UV-radiation to initiate photopolymerization. PEGDMA microvessels were collected in PBS and stored at 37 °C overnight in PBS to remove excess PEG-400, un-reacted macromers, and photoinitiator. To verify uniformity of each synthesis, sections of the microvessels were dried and characterized by scanning electron microscopy (not shown).

Design and fabrication of perfusable microvessel manifold for diffusion studies

The manifold was designed using Autodesk Inventor Professional 2015 (Autodesk, Mill Valley, CA) and was composed of two panels. The bottom panel consisted of the cell-culture chamber, retaining walls, and distribution reservoirs. Typical cell-culture chambers were on the order of 1 cm $\times$ 2 cm $\times$ 0.5 cm, and the distribution reservoirs were triangular with an apex of 0.5 cm. The top panel consisted of matching retaining walls and a glass viewport. Manifold devices were printed using an Objet500 Connex3D Printer (Stratasys Ltd., Edina, MN) with MED610 biocompatible ink.

To assemble the manifold device, PBS-saturated PEGDMA microvessels were placed across the cell culture chamber and retaining wall which separates the cell culture chamber and distribution reservoirs (Figure 1(c)). The cell culture chamber containing the PEGDMA microvessels was filled with a solution of 5 wt. % GelMA and 0.1 wt. % I2959. To hold the microvessels in place, distribution reservoirs were filled with Novagard. All chambers were solidified by exposure to UV-radiation (365 nm, 100 mJ cm^{-2} for 30 s). Approximately 2 mm of silicone within the reservoirs were excised to create unobstructed access to the microvessels. Vacuum grease was lightly spread on one side of the completed manifold device and the top was placed and sealed using screws and nuts. A glass slide was adhered above the viewport with optical adhesive. Fluid was flowed through the device using a programmable peristaltic pump (World Precision Instruments Sarasota, FL). A step-by-step fabrication procedure with photographs is provided in the [supplementary material](#) (Figure S3).

Numerical simulation of fluid flow in the manifold device

Numerical simulations were performed using finite-element analysis software (COMSOL Multiphysics[®], Burlington, MA). The fluid flow profiles were modelled for a single microvessel embedded in a permeable matrix. The model geometry is shown in supplementary Scheme S1. Fluid containing 1 μM “nutrient” was supplied from a single inlet at varying flow rates. The parameters for density, viscosity, porosity, permeability and diffusivity in the media, microvessel wall, and cell-culture matrix are summarized in supplementary Table S1. The simulations accounted for a primary flow through the microvessel, Q_{lumen} , and secondary flows through the microvessel wall, Q_{wall} , and permeable matrix, Q_{matrix} . Fluid flow through the microvessels is

FIG. 1. (a) and (b) Schematic of the Microvessel Manifold Device. The device consists of an inlet and an outlet leading to distribution reservoirs (pink) separated by a central cell culture chamber (yellow). Raised manifolds form the side of the culture chamber (seen in white in top image) and are used to align and hold the microvessels. (a) Media is flowed through the inlet and pushed through the microvessels towards the outlet. (b) From the distribution reservoirs, media or other compounds can be pumped through the manifold of synthetic microvessels to supply nutrients, remove waste, collect analytes, or introduce controlled shear and pulsatile forces to the 3D cell culture. (c) A representative microvessel manifold device with integrated PEGDMA microvessels. Notice the pillars between the inlet/outlet reservoirs and the tissue culture chambers. The pillars provide for quick alignment and positioning up to nine microvessels. (d) Representative photographs of the microvessel manifold removed from the microfluidic device showing suspended microvessels aligned across what would be a 3D tissue culture. (e) A scanning electron micrograph of a PEGDMA microvessel.

assumed to be laminar and obey steady-state Navier-Stokes equations. Fluid flow through the microvessel wall and cell-culture matrix was assumed to obey Darcy's Law. Porosity of the microvessel wall was estimated based on the percent of PEGDMA utilized in making the microvessels.^{33,34} Porosity of the permeable matrix was estimated from published values for polymer volume fraction in swollen hydrogels of GelMA.^{35,36} The microvessel walls and cell culture matrix were assumed to be completely swollen; therefore, the mechanical effects of swelling on solute diffusion are neglected. It is assumed the hydrogel does not contain any significant volume of blind pores. The silicone manifold was treated as a no-flux boundary. The effect of varying the inlet fluid velocity and the porosity of the microvessel wall was simulated by parametric sweeps of the aforementioned parameters.

Diffusion experiments

Diffusion across the interfaces of the lumen, microvessel wall, and GelMA matrix was characterized using digital microscopy to monitor the concentration of trypan blue diffusing from the lumen, through the PEGDMA microvessels and into the GelMA matrix. For the diffusion experiments, a reservoir of 1 μM trypan blue solution in deionized water was connected to the manifold device's distribution reservoir through a peristaltic pump. Images of the cell culture chamber were captured every 10 s for 1 min as the pump began to introduce the trypan blue solution into the device, and subsequent images were recorded every 10 min for the next 9 h. The 10 s interval between the initial set of images provided precise identification of the time when the dye solution first enters the device. The peristaltic pump delivered the trypan blue solution at $185 \mu\text{l min}^{-1}$.

Three sections (near the inlet, middle, and near the outlet) along the PEGDMA microvessels were selected and defined as regions for data analysis. The recorded image sequences were imported into ImageJ (NIH, <http://imagej.nih.gov/ij/>). The color channels in the sequence were split, and the red channel was chosen for analysis. A pixel-to-distance conversion factor was found by measuring an observed region in microns and determining the number of pixels that span the same field of view.

A measurement line was drawn across the field of view from the center of the microvessel lumen, through the wall, and 2 mm into the surrounding matrix. A diffusion profile was generated for each time point by plotting the gray value against distance in microns. To eliminate any variations due to reflection in the manifold, the profile generated before any trypan blue was introduced was subtracted from subsequent image profiles. The trypan blue position at each interval was determined by having a gray value greater than 30. Intensity versus position data was extracted and quantified. The grayscale intensity and dye concentration were correlated by a calibration curve.

Integration of synthetic microvessels in 3D cell culture

An alternative manifold device was utilized for 3D cell-culture experiments. A negative mold of the manifold device was fabricated by direct milling features into 6061 aluminum. Silicone castings of the manifold were produced and sterilized by autoclave. Primary dermal fibroblasts were grown to confluence in complete media (FBM containing growth supplements and gentamicin-amphotericin). The cells were collected via centrifugation after a 5 min incubation in trypsin-EDTA at 37 °C. Cells were re-suspended in 5 wt. % GelMA with 0.5 wt. % I2959 to a density of 1×10^6 cells·ml⁻¹. Microvessels were placed in the device spanning the cell culture chamber and extending into each distribution reservoir. The cell culture chamber was filled with fibroblast suspension and the entire device was irradiated for 30 s (365 nm, 100 mJ·cm⁻²). There was no significant decline in viability due to UV radiation ($p = 0.7959$, supplementary Figure S5).

Glass slides were spin coated with polydimethylsiloxane (PDMS) (1500 rpm for 30 s) and thermally cured to produce a thin PDMS layer. The coated slide and manifold were sealed by selective surface activation with a handheld corona treater (ElectroTechnicProducts, Chicago, IL). This permitted the sealing of the device after the addition of the microvessels and cell matrix. The disposability of the manifold devices facilitated sterility and replication of experiments. A step-by-step fabrication procedure with photographs is provided in the [supplementary material](#) (Figure S4).

Characterization of cell viability

To perform viability assays, manifold devices were disassembled and assayed using LIVE/DEAD cell viability assay (Thermo Fisher). Briefly, tissue constructs were incubated in calcein-AM (2 μM) and ethidium bromide (4 μM) according to manufacturer's recommendations. Calcein-AM fluorescence (green) indicated live cells, whereas ethidium bromide fluorescence (red) indicated dead cells. Z-stacked micrographs were collected and representative images were used to quantitate live (green) cell versus dead (red) cell populations present within the tissue matrix. Plots depict counts of >500 cells per condition. Statistical analyses were performed using the analysis of variance (ANOVA) or student's t-test.

Phalloidin cell staining was imaged similarly and used to identify characteristic fibroblast spreading, typical of cells undergoing expansion. For phalloidin immunostaining, manifold devices were similarly disassembled; tissue constructs were fixed in 4% neutral buffered formalin for 2 h, at room temperature and then permeabilized in 1X PBS containing 0.5% Triton-X100 for 1 h at room temperature. Fibroblast tissue constructs were then incubated in Alexa-488 phalloidin (Thermo Fisher) at 1:40 dilution for 3 h at 4 °C and imaged using a confocal microscope (Nikon A1R).

RESULTS AND DISCUSSION

Perfusable microvessel platform

The integration of microvessels within 3D cell cultures provided a route for small molecules, both nutrients and waste, to be transported to and from cells distributed within the extracellular matrix. Several vascularized organ-on-a-chip devices have been reported; however, the presented design incorporated customizable, free-standing microvessels that mimic the structure and function of blood vessels. The constant directional flow of reagents encouraged two-way passive diffusion of

small molecules into regions of lower concentrations, and therefore the exchange of nutrients and waste. Figure 1 depicts the assembled perfusable microvessel manifold, and the 9-microvessel and 3-microvessel devices are shown in Figures 1(c) and 3, respectively. The microfluidic components were composed of a cell-culture chamber and two distribution reservoirs separated by a manifold containing equally spaced indentations. During encapsulation, the manifold held the microvessel within the center of the GelMA and ensured reproducibility and homogeneity of the environment by maintaining a consistent vessel to vessel distance.

First, microvessels were fabricated by a microfluidic fabrication system using cytocompatible hydrogels. The microfluidic fabrication system provided a means for producing meter-long photoinitiated polymeric microvessels with tunable morphology and physicochemical constituencies.^{28,32} The mesh size and density of the hydrogel can be customized based upon the initial macromer chain length and concentration. Furthermore, adjustment of the macromer composition and flow rates within the microfluidic shaping device can be used to precisely tune the radius of the lumen, porosity, and wall thickness.^{32,37} PEGDMA was chosen as a macromer for microvessel synthesis because it is a robust and transparent hydrogel that is biocompatible and permeable to gases and nutrients. These characteristics are commonly preferred in a variety of biomedical applications, including artificial tissue scaffolds and matrices for the controlled release of biomolecules.^{38,39}

For the demonstration of the perfusable microvessel manifold, PEGDMA microvessels were produced with constant dimensions and either 50% or 80% initial macromer concentrations. Scanning electron microscopy was used to verify the dimensions of the microvessels.²⁸ The PEGDMA microvessels had a total diameter of 275 μm with an inner diameter (I.D.) of 125 μm and a wall thickness of 75 μm . The inner diameter, outer diameter, thickness, and chemical constituency of the walls can all be independently adjusted as desired, and this has been previously reported.^{28,31,37} Each of these parameters will ultimately affect the transport of material from the perfusing media into the tissue matrix. The selected geometry of the microvessels was analogous to the diameters of arterioles and venules—the microvasculature that branches from arteries and veins. Recapitulation of vasculature on this scale is critical for mimicking the vascular network present in a majority of human tissue.

Modelled delivery of nutrients in manifold device

Fluid flow and associated shear forces affect cell growth and differentiation.^{40–43} Interstitial flow also affect the extracellular matrix and growth factors which lead to changes in surrounding tissue.^{44–46} Accordingly, we simulated both the fluid flow and diffusion profiles through the microvessel manifold. The results of the simulations are presented in Figure 2.

For all simulations, a simplified geometry was used. Supplementary Scheme S1 illustrates the simplified geometry of the lumen, microvessel wall, and matrix, and these regions correspond to domains (i), (ii), and (iii) presented in Figure 2, respectively. Taking advantage of the symmetry of the microvessel manifold, only half of a microvessel was modeled. The system was assumed to contain an incompressible Newtonian fluid, eliminating any changes in viscosity and qualifying the assumption of Navier-Stokes flow. A detailed explanation of the physics models utilized to simulate both the fluid flow and diffusion are provided in the [supplementary material](#). In summary, fluid velocity fields were modeled by Darcy's Law and a modified Brinkman equation to account for the flow through the porous media. The diffusion of small-molecule solutes was modeled by Fick's law with an adjustment for the tortuosity of the hydrogel matrix and taking into account the convective flux caused by the fluid flow. Initial material parameters for the simulations are provided in supplementary Table S1.

Figure 2(a) shows the results of the velocity field simulation for a starting inflow condition with a volumetric flow rate of 185 $\mu\text{l min}^{-1}$ introduced through the lumen. Poiseuille flow velocities occur through the length of the lumen but quickly diminish in the porous wall of the microvessel as well as the GelMA matrix. Convective flow exists approximately 125 μm into the wall of the microvessel, but it does not appreciably extend into the surrounding matrix.

FIG. 2. Flow Simulations in Microvessel and GelMA Matrix. Simulations were performed using the geometries depicted in supplementary Scheme S1, with a line of symmetry longitudinally through the lumen. (a) The lumen ((i), bottom of model), the microvessel wall ((ii), PEGDMA), and the tissue chamber ((iii), GelMA) were treated as porous matrices allowing fluid to pass through the interface (left). An inlet was set at the beginning of the lumen with an outlet set at the end of the lumen. Flow is laminar along the center of the lumen but also merges into the vessel wall and into the GelMA. Inlet velocities varying from $10 \mu\text{L min}^{-1}$ to $100 \mu\text{L min}^{-1}$ were simulated. The increase in inlet velocity only marginally affected convection within the wall and was negligible in the GelMA, as shown in the plot (right). (b) The diffusion of the trypan blue was simulated at 30 min intervals for 10 h. There is a high concentration of the solute within the wall immediately following introduction, while the diffusion of the solute throughout the hydrogel matrix takes several hours to become linear. For data plots, each line represents either the (a) velocity or (b) concentration from 1 to 10 h, in 1 h intervals.

FIG. 3. Micrograph demonstrating typical diffusion observed in a 3-microvessel manifold device with 80% PEGDMA microvessels sealed inside. (a) Initially ($t = 0$ h), trypan blue flows through the inlet (left reservoir), through the microvessels, and into the outlet (right reservoir). The photograph was taken within minutes of introducing flow through the microvessels. The trypan blue diffusion is retarded by the PEGDMA mesh and has not yet reached the pristine GelMA matrix. (b) Following 8 h, the trypan blue has diffused through the microvessel wall and into the GelMA matrix.

Flow rates ranging from 50 to 500 $\mu\text{L min}^{-1}$ were simulated and no remarkable changes in convection within the wall or matrix were observed.

Figure 2(b) shows the results of the diffusion and convection simulations based on the previously simulated velocity fields and an inflow of 1 μM “nutrient.” The simulations show a quick increase in concentration within the wall of the microvessel, which has a higher intrinsic diffusivity, suggesting that diffusivity governs the transport. A slower increase in nutrient concentration occurs throughout the GelMA scaffold.

The porosity of the microvessel walls was also varied to model the afforded control of nutrient and waste perfusion through the manifold device that is gained by using a modular microvessel platform (Figure S2). The PEGDMA concentration can easily be changed during fabrication which would affect the porosity of the microvessel wall. Increasing the pore size results in increased rate of nutrient transport across the microvessel wall. The ability to fine tune the microscale morphology and chemical constituency of the microvessels before embedding in the manifold device enables end-users to control the rate and gradient of material transport into the cell culture chamber to mimic native tissue.

Empirical diffusion measurements in manifold device

Several biosynthetic and synthetic extracellular matrix (ECM) hydrogels are available commercially and can be supplemented to include many components found *in vivo* such as structural proteins, proteoglycans, and growth factors. However, these products often vary from lot to lot and are heterogeneous in composition.⁴⁷ An ECM's bulk diffusion properties are governed by both its cross linking density^{48–50} and stiffness,^{51,52} which are dependent on the concentration of proteins that polymerize during scaffold formation. GelMA was used as a structural matrix because it offers a convenient method for providing a tissue scaffold with customizable homogenous viscoelastic properties.³¹ Furthermore, the GelMA backbone offers many opportunities for chemical modifications, thereby enabling future optimization and customization of the physiochemical properties of the hydrogel.

GelMA has previously been shown to be an effective cellular matrix in its unmodified form at a range of concentrations, with 5% being able to sustain high cell viability.⁵³ Each diffusion experiment used a constant GelMA and photoinitiator concentration of 5% and 0.05%, respectively. Microvessels composed of either 50% PEGDMA or 80% PEGDMA were arranged within the GelMA and sealed. Trypan blue (872.88 g mol^{-1}) was chosen as a model compound to trace the diffusion from the luminal flow into the surrounding matrix. It has a larger hydrodynamic radius and molecular weight than basal media nutrients, e.g., oxygen, glucose, vitamins, essential amino acids, and glutamine; therefore, it is a suitable “cut-off” model for diffusion of critical nutrients. In addition, Trypan blue has been used as a common model for charged drug species in hydrogel diffusion and controlled release studies.^{54–58} Recently, it has even been used to analyze cell viability *via* perfusion through hydrogels into the bulk tissue of the brain-on-chip model.⁵⁹ Due to its diffusion properties, broad use as a model drug and high extinction coefficient which enables the easy imaging of diffusion gradients, we selected Trypan blue as the model solute to characterize diffusion from the perfused media, across the microvessel wall and into the cell culture chamber (Figure 3).

The diffusion of Trypan blue from the lumen of the microvessel, through the wall, and into the GelMA was monitored over several hours (Figure 4(a), supplementary video S1). Both microvessel types showed the same general pattern that was seen in the simulations. Microvessels consisting of 50% PEGDMA showed a broad diffusion gradient with a high localized concentration at the microvessel lumen (Figure 4(b), top). Conversely, microvessels consisting of 80% PEGDMA show a higher intensity within the wall, and a more acute decline beyond the wall (Figure 4(b), bottom). This is seen clearly in Figure 4(c) where the diffusion front spreads nearly three times as far when diffusing from microvessels consisting of 50% PEGDMA than when diffusing from microvessels consisting of 80% PEGDMA (1500 μm vs 500 μm). This behavior is similar to membrane concentration polarization, wherein select molecules do not transfer through membrane pores and thus create a concentration gradient along

FIG. 4. Passive Diffusion through Microvessels. PEGDMA microvessels were synthesized and incorporated into manifold with pristine GelMA within the cell culture chamber. Trypan blue introduced into the device (at $t = 0$). (a) Diffusion of trypan blue was monitored over the course of several hours by time-lapse imaging. The density of trypan blue within the wall is higher when using a higher macromer concentration due to a concentration polarization effect. (b) Trypan blue passively diffused from the lumen (i) into the wall (ii) and into the pristine GelMA (iii) creating a concentration gradient. The intensity of the trypan blue gradient was normalized in relation to the intensity of the initial inflow. Each line describes the diffusion gradient at a different point in time at 1 h intervals. (c) The position of the diffusion front was determined by observing the point where the grey value rose above background at each time interval. While the concentration within the wall of the 80% microvessel was more intense, the decreased mesh size limited diffusion of the solute to the extremities of the GelMA matrix.

the span of a filter.⁶⁰ As the macromer concentration increases, pore size decreases, and consequently so does the sieving efficiency of the microvessels. These results suggest that a lower initial macromer concentration positively affects the net flux of molecular species into and out of the system. Similar effects have been reported for the diffusion of small-molecule dyes from flow through poly(ethylene glycol) diacrylate gels.⁵⁴ Based on these results, we can foresee the manifold device being used to evaluate the diffusion and transport characteristics of many solutes, e.g., growth factors, small-molecule drugs and nanomaterials, across a microvessel wall with a desired chemical constituency and delivered into the surrounding tissue culture.

Incorporation of synthetic microvessels in 3D cell culture

The primary purpose of the perfusable microvessel manifold presented here was to efficiently limit necrosis of the cells due to hypoxia, metabolic waste accumulation, and inadequate nutrient diffusion. Initial experiments were conducted to determine the cytocompatibility and structural stability of the microvessels within a populated GelMA matrix over several days. We found that, while 3D printed manifolds worked well for diffusion experiments, they were difficult to sterilize and had poor cytocompatibility. To alleviate this problem, a replica of the 3D printed device was cast out of PDMS using an aluminum mold master (see Materials and Methods). Following thermal polymerization, the manifolds were thoroughly sterilized in 70% ethanol. Traditionally, PDMS is irreversibly secured to glass through use of a plasma asher.⁶¹ However, due to the hydrophilic nature of our microvessels, pre-treatment in a plasma cleaner

FIG. 5. Encapsulation of Microvessels in Fibroblast Laden Hydrogels. Microvessels were synthesized and encapsulated in a device containing a suspension of fibroblasts in 5% GelMA. Optical micrographs show the embedded microvessels (a) immediately after embedding and after (b) 7 days of culture. The devices were fixed and actin filaments were stained using phalloidin (c) immediately following embedding or (d) 7 days following culture. At day 7 fibroblasts (green) proliferated within the biosynthetic extracellular matrix and displayed morphology consistent with cell spreading. A steady flow of fluorescent microparticles (red) were passed through the microvessels with no visible leakages or structural instabilities. A 3D reconstruction was made using confocal microscopy with a $6\text{ }\mu\text{m}$ step size. Fibroblasts are seen lining and surrounding the microvessel.

caused the microvessels to adhere to the walls of the device and the GelMA suspension to leak into the newly wetted reservoirs. Thus, selected areas of the device were activated using a handheld corona treater. This method produced a leak-free seal following the microvessel incorporation and polymerization of the extracellular matrix.

Fibroblasts were cultured for 7 days with daily renewal of the media in the peristaltic reservoir. Using fluorescently labeled phalloidin (green) along with a DAPI counterstain (blue), we imaged the system while flowing fluorescent microparticles (red) through the lumen (Figure 5). The fibroblasts show a consistent morphology and the high cell density suggesting very little loss in viability due to nutrient deprivation. Furthermore, the structural stability of the microvessels was also maintained throughout the culture period with no detriment in flow efficiencies and no evident leakages of the microparticles into the GelMA matrix (Video S2). As a static control, fibroblasts were cultured in the manifold device without embedded microvessels and subsequently no perfusion of media. The fibroblasts showed no proliferation, no morphology indicating spreading, and significantly reduced viability after 7 days (Figures S6 and S7).

CONCLUSIONS

Incorporation of synthetic blood vessels into organ-on-chip models is a viable utility for overcoming hurdles such as hypoxic local environments, excessive effluent accumulation, and inadequate nutrient perfusion. The presented manifold system represents an alternative method

for utilizing synthetic blood vessels to vascularize organ-on-chip models. The ability to incorporate free-standing synthetic microvessels and deliver media throughout the 3D cell culture opens a wide-range of applications for the ongoing development of microfabricated blood vessels and enables the engineering of larger tissue constructs with controlled and consistently placed microvasculature.

We have demonstrated, through the use of a nutrient analog, the capability of the synthetic microvessels to deliver chemical species through a thick tissue matrix distance of greater than 1 mm. Furthermore, we have proven the usefulness of our approach by using the microvessels to deliver nutrients to a thick 3D cell culture matrix. While the presented device uses acellular microvessels, the fabrication process can be adjusted to incorporate macromers that provide a method to incorporate resident cells and enhance cellular adhesion within the lumen of the microvessels.⁶² We plan to pursue complex vascular tissue constructs in future research.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for parameters of diffusion simulations and results, step-by-step fabrication protocols for manifold devices, results of cell viability assays, and videos of manifold devices during operation.

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