METHODS ARTICLE

In Vivo Anastomosis and Perfusion of a Three-Dimensionally-Printed Construct Containing Microchannel Networks

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The field of tissue engineering has advanced the development of increasingly biocompatible materials to mimic the extracellular matrix of vascularized tissue. However, a majority of studies instead rely on a multiday inosculation between engineered vessels and host vasculature rather than the direct connection of engineered microvascular networks with host vasculature. We have previously demonstrated that the rapid casting of three-dimensionally-printed (3D) sacrificial carbohydrate glass is an expeditious and a reliable method of creating scaffolds with 3D microvessel networks. Here, we describe a new surgical technique to directly connect host femoral arteries to patterned microvessel networks. Vessel networks were connected *in vivo* in a rat femoral artery graft model. We utilized laser Doppler imaging to monitor hind limb ischemia for several hours after implantation and thus measured the vascular patency of implants that were anastomosed to the femoral artery. This study may provide a method to overcome the challenge of rapid oxygen and nutrient delivery to engineered vascularized tissues implanted *in vivo*.

Introduction

TISSUE ENGINEERING EMPLOYS material-based bioengineered constructs containing living cells that can be used for tissue replacement therapy.^{1–3} Previous studies have demonstrated that growth factors and molecular signaling, together with mechanical and electrical stimulations, are key components in the successful growth of tissue *in vitro*.⁴ Ideally, the scaffold should be biocompatible, biodegradable, and minimally immunogenic.¹ One of the major challenges in tissue engineering is the selection of a delivery method that optimizes cell retention, survival, and differentiation. Currently, common methods for delivering cells or tissue grafts *in vivo* include the intravenous injection of cells, injection of cells within a hydrogel carrier, avascular implantation of the graft, or implantation of the graft with the anastomosis of host vasculature.^{5,6} A delivery system must ensure cell viability and support cellular integration with the target tissue. When successfully engineered, constructs should provide direct, sustained mechanical support and molecular cues to promote angiogenesis along with cell engraftment, differentiation, maturation, and integration with the host tissue.^{7,8}

Adequate graft vascularization and efficient integration with host circulation are paramount in translating engineered tissues into clinically relevant therapies.⁹ Engineering a thick and complex gel that can sustain a cell population requires a highly organized, built-in vascular network. Many research groups have turned to three-dimensional (3D) printing as a method to generate complex vascular structures in large-scale engineered tissues. 3D printing offers researchers control over every position within the print volume and allows the specific placement of multiple materials or cell types. Groups are employing many varied approaches using a variety of 3D printing techniques to fabricate vasculature, including extrusion printing, photolithography techniques, laser sintering, and sacrificial extrusion printing.^{10–13}

Although tremendous effort has been placed on studying new 3D scaffolds among other novel engineering techniques to enhance cell survival and integration, little effort has been placed on a transplant surgery model for graft implantation.² Currently, most techniques for implanting prevascularized tissue constructs rely on the ingrowth of host vessels and wrapping and tapping anastomosis for perfusion of the engineered vasculature.^{14–17} This method of anastomosis has been shown to take from a few days to weeks to perfuse the

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implanted vascular network and appears to be highly dependent on the geometry of the microvascular network.^{17–19} However, this time frame may be too slow to ensure the viability of cells within constructs containing cell densities approaching those of native tissues such as the human liver.²⁰ Furthermore, although other groups focusing on the implantation of whole organs, such as organ transplants or recellularized whole organs, must connect the original vascular structures to those of the host tissue, these approaches depend on the native structure of the implanted organ and lack some of the desired control of engineered constructs.^{21,22} To address the issue of an implantation strategy to rapidly perfuse prevascularized networks, we have developed early surgical strategies through which perfusion could be maintained through 3D-printed vascular networks. Following implantation, we are using a modified hind limb ischemia model to assess the patency and flow of gels containing 3D-printed vascular networks following *in vivo* implantation.

The ability to develop an internal microvascular network within a hydrogel and connect the microvasculature into the host vascular network is an essential step toward generating large vascularized grafts with cellularized interstitial in host tissue. In this study, we engineered and assessed the *in vivo* patency of polydimethylsiloxane (PDMS) constructs with built-in microchannel networks that mimic an integrated microvascular network. We hypothesized that the microvascular tree within the hydrogels could be successfully perfused in series with the native circulatory system. Using

our new surgical technique to implant these gels in line with the femoral artery, we used a modified hind limb ischemia model to assess the perfusion and patency of the channels over time. As a proof-of-concept demonstration, we have established a workflow to rapidly perfuse the internal vasculature of 3D-printed vascularized constructs implanted *in vivo*.

Materials and Methods

PDMS gel with microchannel fabrication

PDMS gels were generated as described previously, using 3D printing of sacrificial sugar glass. Briefly, sugar-glass lattices were printed using a ShopBot Desktop D2418 Router (ShopBot, Durham, NC) modified to hold an extrusion print head. Lattices were printed using the geometry shown in Figure 1, which consists of layered wells surrounding and supporting two primary vessels (~1 mm in diameter) on top of four secondary vessels (~300 μ m in diameter). After the lattices cooled, the ends of the cross-channels stretching between the primary channels and the well wall were removed using a fine-tipped soldering iron. In addition, one end of each of the main vessels was removed to generate a structure with a single inlet and outlet, as shown in Figure 1. The sugar-glass lattices were then cast in Sylgard 184 PDMS (Dow Corning, Midland, MI) by filling the printed well with 20:1 PDMS:curing agent mixture. The PDMS was then allowed to cure for two days.

FIG. 1. Description of sugar-glass printing and initial flow testing. (A) Extrusion print head in the process of printing a sugar-glass lattice. Print head can move x, y, or z planes. (B) Final sugar lattice before casting. The lattice contains a network of filaments supported by a surrounding well. Red line denotes the outer edge of the well that will be filled with polydimethylsiloxane (PDMS) during casting. (C) Schematic of printed sugar-glass network. Drawing on the left denotes sugar filaments after printing, whereas the figure on the right shows filaments before casting. Unwanted filaments are removed using a fine-tipped soldering iron before casting. (D) Final PDMS gel with channel network. Excess PDMS has been removed using a razor blade. (E) Microcomputed tomography reconstruction of internal channel network from a cast PDMS construct. (F) Computational model of flow rates through cast channel geometry. Flow streamlines are color coded corresponding to flow rate. Flow rate at the inlet is equal to 0.12 mL/min. Computational models demonstrate the continuity of flow and patency of channel networks. Scale bar=4 mm. Color images available online at www.liebertpub.com/tec

Following curing, the sugar glass was removed from the PDMS by placing the PDMS/sugar-glass constructs into a solution of 30% isopropanol in deionized water for 2 days, replacing the isopropanol/water solution every 12 h. To reduce the size of the final construct, the edges of the PDMS gels (not containing any channels) were removed using a razor blade.

Microcomputed tomography scanning and computational flow analysis

Microcomputed tomography (micro-CT) scans were performed on PDMS gels ($n=4$) to obtain 3D models of the internal channel networks. The scans were conducted using a Bruker SkyScan 1272 at the Baylor College of Medicine Optical Imaging and Microscopy Core. The source voltage was set to 70 kV, with a source current of 142 μ A, an exposure time of 2952 ms, and a rotation step of 0.5 degrees. The scans had a nominal resolution of 6 μ m. Using the NRecon software (Bruker, Kontich, Belgium), the scans were then reconstructed, correcting for misalignment and ring artifacts. The reconstructed image stack was then filtered using a 3D Gaussian filter with a width of 5 pixels. Using the Mimics Innovation Suite software (Materialise, Plymouth, MN), pixel values were then inverted and thresholded to generate a clear surface for the internal channel network. Small unconnected objects smaller than 50 μ m were removed. The image stack was then saved as a 3D model in a stereolithography (.stl) file. The mesh was adjusted to improve element quality and remove any holes or inverted elements (Fig. 1).

To estimate flow through the structure, the reconstructed channel geometries were imported into COMSOL Multiphysics 5.0. Using the single-phase laminar flow module, the inlet and outlet of the channels were defined under no slip conditions. The inlet flow was defined as 0.12 mL/min with no pressure drop between inlet and outlet.²³ The resulting flow rates were plotted using streamlines where color corresponds with the flow rate (Fig. 1).

Animal care and biosafety

Male Wistar rats weighing 400–450 g were obtained from Charles River Laboratories International, Inc. (Wilmington, MA). All animals were provided with water and food ad libitum and maintained in a climate-controlled environment accredited by the Institutional Animal Use and Care Committee of the University of Pennsylvania. All animal experiments were in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (Eighth Edition, 2011).

PDMS gel implantation

Ten male rats were evenly divided into a positive control and a negative control group. In the positive control group, the PDMS gel was implanted in the right common femoral artery and the left common femoral artery was left intact. In the negative control group, the PDMS gel was also implanted in the right common femoral artery, but the left common femoral artery was double ligated and transected proximally. Each rat underwent general anesthesia with a mixture of 4% isoflurane with 100% oxygen

in a 2-L induction chamber (VetEquip, Pleasantville, CA). Once sedated, the animals underwent endotracheal intubation with a 16-gauge (G) angiocatheter (Tyco Healthcare, Mansfield, MA) and were ventilated at a rate of 80 breaths per minute and tidal volume of 30 cc. The hind limbs of the animals were positioned with silk bandages. Bilateral inguinal areas of the animals were clipped and prepped in a sterile manner. A 3 cm incision was made in the right infrainguinal region in both groups. Corresponding 3 cm incisions were made on the contralateral hind limb of the negative control group. No surgical operations were undertaken in the left hind limbs of the animals in the positive control group.

The entire procedure was performed under a Leica M60 modular stereomicroscope (Leica Microsystems, Inc., Buffalo Grove, IL). The incision was taken through the subcutaneous tissue until the femoral sheath was exposed (Fig. 2A). The femoral artery was separated from the femoral vein and nerve (Fig. 2B). PDMS gels were implanted in the right groins of all the animals in the experiment. Control of the proximal and distal femoral artery was obtained by placing a 5-0 silk suture (Ethicon, Somerville, NJ) around the vessel. The proximal and distal femoral arteries were cannulated in a stepwise manner using two separate 26G angiocatheters and secured in place by the silk sutures (Fig. 2C–E). One hundred units of heparin (Sagent Pharmaceuticals, Schaumburg, IL) was injected into the systemic circulation using the proximal catheter. The PDMS gels were flushed with 10 cc of heparinized saline *ex vivo* to assess flow. Next, using a pair of fine hemostatic clamps, the proximal end of the femoral artery was temporarily clamped, the segment of artery between the two catheters was transected, and the 26G angiocatheters were trimmed to accommodate the length of the PDMS gel without any kinks along the system. The trimmed tip of the proximal angiocatheter was carefully mounted onto the proximal inlet of the PDMS gel microchannel, and the proximal hemostatic clamp was temporarily removed to assess pulsatile flow through the gel (Fig. 2F). The distal angiocatheter was mounted onto the distal outlet of the PDMS gel and the hemostatic clamp was released (Fig. 2G). Flow and pulsatility through the microchannel of the gel were assessed visually and using Doppler imaging technology. In the negative control group, the left femoral artery was double ligated and transected proximally.

Flow measurement

Blood flow through the gel microchannels was assessed using a Moor Laser Doppler Imager LDI (Moor Instruments, Inc., Wilmington, DE) immediately, 1 h, and 3 h postgel implantation. Moor LDI software V.5.3 (Moor Instruments, Inc.) plots the relative flow rates as a colored heat map (Fig. 3). Before any imaging, each rat was injected with 10 cc of normal saline subcutaneously around the abdomen to increase intravascular volume and account for blood loss and insensible losses during the operations. The animals were kept ventilated under general anesthesia (2% isoflurane; 100% oxygen) for the duration of the Doppler imaging studies. In between flow measurements, the animals were kept on a warming platform and wrapped in a blanket to prevent hypothermia.

FIG. 2. (A) Incision and exposure of femoral neurovascular bundle. (B) Arrows showing femoral nerve (n), common femoral artery (a), common femoral vein (v) dissected, and skeletonized *in situ*. (C) Cannulation of proximal common femoral artery with 26-gauge angiocatheter. (D) The angiocatheter is advanced in the arterial lumen (arrow) with brisk blood flash observed inside the catheter. One hundred units of heparin was injected systemically through this catheter. (E) Arrows showing proximal (p) and distal (d) common femoral artery cannulated and secured with silk suture before transecting the intercatheter arterial segment. (F) The proximal catheter is mounted on the PDMS gel inlet and blood flow tested. (G) The distal catheter is mounted on the distal outlet of the PDMS gel, and all hemostatic clamps were removed. (H) Pulsatile blood flow through the gel microchannels and perfusing the distal hind limb. Color images available online at www.liebertpub.com/tec

FIG. 3. (A) Arrow pointing to site where the femoral artery was ligated and transected, and p indicates the paw of the animal. (B, C) No blood flow to limb. (D–F) Arrows pointing to the PDMS gel with flow through the microchannels. The outline and individual channels of the gel can be seen under Doppler imaging. (G–I) The whole hind limb of the animal has flow. Color images available online at www.liebertpub.com/tec

Results

Computational models of flow through vascularized gels

To assess patency of the channels before implantation, we used micro-CT scans of four different gels to assess the internal channel geometry and generate a 3D computational mesh. We then imported the geometry into COMSOL Multiphysics to assess flow patterns through the channels. Using an inlet velocity 0.12 mL/min and no slip conditions, we determined that for all four gels, each channel received flow at the provided inlet velocity.²³ A representative gel can be seen in Figure 1. Similar computational modeling will be extremely useful for optimizing vascular geometry to maximize cell viability in vascularized gels in future studies.

In situ placement of vascularized gel

Ten gels were successfully implanted. Upon unclamping the proximal hemostatic clamp, antegrade bright red blood flow was observed filling the microchannels of the gel and pulsatile blood flow was observed out of the distal outlet of the gel (Fig. 2D). The distal angiocatheter was mounted on the gel outlet, and pulsatile flow was observed through the distal femoral artery. The constructs withstood the physiologic pressure of the rat femoral system (80–100 mmHg).²⁴

Confirmation of blood flow and patency

The patency and antegrade flow through the gel were assessed with a laser Doppler at four different time points. Figure 3A and B illustrates sample laser Doppler flow patterns in the positive and negative control groups, respectively. Flow through the gel, distal femoral artery, and the paw were similar in the surgery limb and the positive control. There was noticeably more flow through the gel, distal femoral artery, and the paw compared to the negative control limb immediately after, and at 1 and 3 h postimplantation (Table 1).

Discussion

In this study, we have successfully developed a method to implant 3D-printed gels containing microchannel networks into a rat's native circulation for immediate perfusion of the construct. Moreover, we have demonstrated successful flow and biocompatibility with the native circulation. The 3D-printed construct withstood the physiologic pressures of the rat femoral artery at 80–100 mmHg.²⁴ Pulsatile flow through the microchannels was confirmed upon implantation as well as up to 3 h postimplantation. This time frame is similar to other tissue engineering methods incorporating microvessels, such as implanted organs engineered using tissue decellularization and recellularization techniques.^{21,22,25}

Currently, the only available treatment for end-stage organ failure is autologous and homologous transplant. However, the clinical course for organ transplant is often complicated by primary graft dysfunction, donor-related infection, donor-site hematomas, and long-term immunosuppression-related risks of infection and rejection.⁷ Most significantly, a marked limitation in the availability of donor organs severely limits the availability of these organs to patients in need. Advances in tissue engineering could provide a source of tissue grafts, while avoiding issues such as donor-site morbidity, immune rejection, and donor shortage. However, as the field progresses to engineer thicker and more complex cellularized tissues such as the myocardium, liver, or lung, researchers must replicate native vascular architecture to ensure sufficient diffusion of oxygen and nutrients to the cells. Vascularized tissue has three components: (1) a hierarchy of vessels with different lumen sizes containing circulating plasma, (2) endothelial cells that line the vessel wall and regulate transport from the lumen to the extracellular matrix, and (3) an interstitium that contains the parenchymal cells.²⁰ Most cell types can only survive a distance of 200 μ m from the nearest capillary through diffusion.^{15,18} Scaffolds that sustain a cell population will require artificial microvessels to adequately deliver oxygen and nutrients and to clear metabolic wastes.²⁶ Furthermore, the vascular geometry within a tissue, such as branching pattern, angle, and density of the capillaries, varies

TABLE 1. FLOW MEASUREMENT THROUGH THE GEL AND LIMB

	Surgery limb (n=10)/Flux PU			Positive control (n=5)/Flux PU				Negative control (n=5)/Flux PU			
	Mean	SD	Median	Mean	SD	Median	p-Value ^a	Mean	SD	Median	p-Value ^b
Immediate postoperative (n=10)											
Gel	259	79.64	246.3								
Femoral artery	399.33	137.04	438	314.07	44.22	289.9	0.12	115.43	32.47	106.25	0
Paw	192.89	88.18	181.8	295.4	83.87	249.6	0.15	99.66	15.12	99.2	0.01
1 h postoperative (n=9)											
Gel	225.47	72.4	208.3								
Femoral artery	336.69	138.13	367	344.2	90.68	353.85	0.91	123.16	44.39	117.6	0
Paw	134.17	38.07	134.2	287.13	154.16	234.05	0.14	99.76	18.2	92.1	0.04
3 h postoperative (n=7)											
Gel	216.41	216.41	193.8								
Femoral artery	295.71	153.61	316.1	329.98	51.71	318.4	0.6	154.13	90.6	120	0.02
Paw	162.11	85.65	124.7	238.58	84.17	224.95	0.01	132.17	51.16	114.6	0.06

^ap-Value measured by *t*-test of unequal variance between surgery limb and positive control.

^bp-Value measured by *t*-test of unequal variance between surgery limb and negative control.

Flux PU is a measure of the amount of blood flow.

Bold, statistically significant.

PU, perfusion unit; SD, standard deviation.

with the tissue's metabolic and growth profile.¹⁸ These features also determine the success of graft neovascularization and incorporation into the host, and it is therefore essential to optimize vascular geometry before implantation and ensure that the tissue will provide accessible anastomosis sites for integration and immediate perfusion.^{3,27} For these reasons, we have selected 3D printing to generate our microvascular networks. 3D printing is a highly adaptable technique capable of generating a multitude of complex vascular geometries on different scales, which allows researchers to generate vascular structures with viable anastomosis sites and improved vascular geometries for integration with the host.

Our group has previously demonstrated in a rat model that superimposing an infarcted area of the heart with a fibrin gel construct seeded with endothelial progenitor cells significantly improves myocardial contractility and function.²⁸ Translating this therapy to treat heart failure patients will require significantly larger gels that have limited diffusion capabilities restricted to the periphery of the gel. Such constructs will develop necrotic cores without an organized vasculature, assessable anastomosis sites, and successful implantation and integration with the host vasculature.

In the previous work, we successfully pioneered the design and fabrication of a vascularized gel containing a cylindrical network of perfusable channels using a sacrificial template of cytocompatible, 3D-printed carbohydrate glass.²⁰ We used this sacrificial 3D-printing technique to generate channel networks within a variety of matrix materials, including agarose, alginate, poly(ethylene glycol) (PEG), fibrin, and Matrigel. These man-made vascular lumens were then successfully seeded with human umbilical vein endothelial cells (HUVECs) under pulsatile conditions *in vitro*. Human hepatocytes loaded in vascularized gels were found to have superior and more robust metabolic profiles than those loaded in regular gels.²⁰ Furthermore, after 9 days in culture, the HUVECs showed signs of sprouting, which suggests that endothelial cells are capable of generating their own capillary networks between the 3D-printed vascular networks.²⁰

In this article, we have successfully shown a preliminary proof of concept for the rapid integration of vascularized gels into the native circulation. We surgically implanted 3D-printed gels containing microchannel networks into the host vasculature and demonstrated that microchannel size, architecture, cross-channel angulation, and geometry are compatible with physiologic pulsatile flow. The successful *in vivo* implantation of our vascular construct is essential for using complex engineered tissues for regenerative medicine applications, particularly in tissues with a high metabolic activity. The ultrastructure of the vascularized matrix will increase the proximity of cells to capillaries supplying oxygen and nutrients that will promote their viability, differentiation, and growth *in vivo*. In future work, we will incorporate endothelial cells into our vascularized gels to help reduce clotting as blood travels through the gel. Furthermore, in accordance with previous work, we expect to encourage sprouting from our endothelialized channel networks for capillary formation and further improvements to nutrient transport within vascularized constructs. We can also generate channel networks using different adhesive and degradable PEG hydrogels and assess *in vivo* patency using the same surgical implantation model. This study is the stepping stone

to bioengineering and implanting complex, 3D tissues, and potentially total bioartificial hearts among other vital organs.

Conclusion

A rapid casting of 3D-printed sacrificial carbohydrates is a reliable method of making 3D scaffolds with microvascular networks. Using this 3D-printing technique, our study provides a method to overcome the challenge of oxygen and nutrient delivery when engineering complex and thick vascularized tissue. For future work, the use of increasingly biocompatible materials to mimic the extracellular matrix of vascularized tissue will allow for prolonged patency of the channel lumens and better integration of the construct with the host tissue.

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Disclosure Statement

No competing financial interests exist.

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