Part 1

- Read the following article: Miller et al. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues, *Nature Materials* (2012), and answer the following questions. For some questions, you may need to look into referenced papers or outside material for additional information.
  [http://www.nature.com/nmat/journal/v11/n9/full/nmat3357.html](http://www.nature.com/nmat/journal/v11/n9/full/nmat3357.html)

a) (3 points) What are the design requirements for the sacrificial material?

b) (3 points) Figure 1b demonstrates a significant reduction in the optical extinction of the carbohydrate glass for light with wavelengths > 350 nm. This feature is important for one of the biomaterials that they combine with the glass in this demonstration. Which biomaterial is this and why is this important?

c) (2 points) Based on their studies, what relative change in the extrusion nozzle travel speed would be required in order to reduce the filament diameter from 800 µm to 200 µm? (assuming no change in nozzle diameter or extrusion flow rate, i.e. no change in A)

d) (3 points) What does the following sentence mean? "As a measure of cellular function and activity, we examined expression of destabilized enhanced green fluorescent protein (dsEGFP) from a constitutively expressed lentiviral cassette inserted into HEK293T cells."

e) (4 points) The authors used the HEK cells expressing dsEGFP to demonstrate the benefit of channels compared to a slab gel. Briefly describe what experiment they performed and what benefit they demonstrated.
Part 2

- Read the following article: Wu et al. Omnidirectional printing of 3D microvascular networks, *Advanced Materials* (2011), and answer the following questions. For some questions, you may need to look into referenced papers or outside material for additional information.  

f) (2 points) Pluronic F127 (also known as Poloxamer 407) is a triblock polymer, what are the segments and how are they arranged?

g) (3 points) What is the critical micelle concentration (CMC)? What happens above the CMC?

h) (3 points) What is the critical micelle temperature (CMT)? What happens below the CMT?

i) (6 points) The fugitive ink, fluid filler, and reservoir gel are all based on Pluronic F127. What are the differences in these components?

j) (3 points) Using the 200 µm nozzle, what range of microchannel diameters could be formed? How did they control the channel diameter?

k) (2 points) How did they polymerize the overall microvascular structure?

l) (2 points) How did they remove the ink?

m) (3 points) How did they test diffusion from the channels?
Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues

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In the absence of perfusable vascular networks, three-dimensional (3D) engineered tissues densely populated with cells quickly develop a necrotic core. Yet the lack of a general approach to rapidly construct such networks remains a major challenge for 3D tissue culture4–6. Here, we printed rigid 3D filament networks of carbohydrate glass, and used them as a cytocompatible sacrificial template in engineered tissues containing living cells to generate cylindrical networks that could be lined with endothelial cells and perfused with blood under high-pressure pulsatile flow. Because this simple vascular casting approach allows independent control of network geometry, endothelialization and extravascular tissue, it is compatible with a wide variety of cell types, synthetic and natural extracellular matrices, and crosslinking strategies. We also demonstrated that the perfused vascular channels sustained the metabolic function of primary rat hepatocytes in engineered tissue constructs that otherwise exhibited suppressed function in their core.

Living tissues have complex mass transport requirements that are principally met by blood flow through multiscale vascular networks of the cardiovascular system. Such vessels deliver nutrients and oxygen to, and remove metabolic byproducts from, all of the organ systems in the body and were critical to the rise of large-scale multicellular organisms5. Although tremendous progress has been made in the past few decades to isolate and culture cells from native tissues, simple methods to generate tissue constructs populated at physiologic cell densities that are sustained by even the most basic vascular architectures have remained elusive.

To create perfusable channels in engineered tissues, layer-by-layer assembly6–9 has been explored. In this approach, a trench is moulded into one layer such that a second, separately fabricated layer can then be aligned and laminated to close the lid to form channels in an iterative fashion. However, layer-by-layer assembly is slow and results in seams or other structural artefacts throughout the construct while simultaneously placing considerable design constraints on the materials, channels, and cells used during fabrication. Bioprinting10, in which cells and matrix are deposited dropwise, has been developed over the past decade but also is a slow, serial process with limitations on print resolution, materials, and cells. In contrast to these methods, 3D sacrificial moulding11–13 provides an intriguing alternative. Proof-of-concept studies have shown that a network of channels can be fabricated by creating a rigid 3D lattice of filaments, casting the lattice into a rubber or plastic material, and then sacrificing the lattice to reveal a microfluidic architecture in the bulk material. However, 3D sacrificial moulding of perfusable channels has so far required the use of cytotoxic organic solvents or processing conditions for either removing the sacrificial filaments or casting the surrounding material, and thus could not be accomplished with aqueous-based extracellular matrices (ECMs) or in the presence of living cells.

Here, we describe a biocompatible sacrificial material—a simple glass made from mixtures of inexpensive and readily available carbohydrates—and a means to print the material to facilitate the rapid casting of patterned 3D vascular networks in engineered tissues. This carbohydrate glass formulation was developed specifically to accommodate two seemingly opposing design criteria that we identified for biocompatible 3D sacrificial materials: sufficient mechanical stiffness to physically support its function inside a tissue, it is compatible with a wide variety of cell types, synthetic and natural extracellular matrices, and crosslinking strategies. We also demonstrated that the perfused vascular channels sustained the metabolic function of primary rat hepatocytes in engineered tissue constructs that otherwise exhibited suppressed function in their core.

Carbohydrate glass can be formed by dissolving one or more carbohydrates in water and then boiling off the solvent. Our early experiments were based on a sucrose–glucose mixture developed by the food industry, which showed that although sucrose is unstable in supersaturated solutions, the addition of glucose prevents recrystallization and facilitates the formation of a stable and inexpensive glass14. This simple mixture was too hygroscopic and soft to handle. During material optimization and screening of potential additives, we found that the addition of starch stiffened the base material, but it imparted inferior optical clarity and therefore limited potential use with matrices that are commonly crosslinked by photochemical reactions15,16. In contrast, the addition of glycerol preserved clarity but rendered filaments mechanically unstable at room temperature. Ultimately, we further reinforced the glass and improved its temperature stability by incorporating dextran. Uniaxial compression testing confirmed that the carbohydrate glass is mechanically stiff and brittle at room temperature (Fig. 1a).

Multiscale vascular networks comprise a range of diameters of vessels and their interconnections. Thermal extrusion and fibre drawing with a 3D printer—a programmable Cartesian coordinate positioning system—provided an effective route to the fabrication of filamentous carbohydrate glass lattices. By
Figure 1 | Carbohydrate-glass material properties and filament-architecture formation. a, Stress-strain curve from uniaxial compression testing indicates that the carbohydrate glass is a stiff and brittle material at 25 °C, with Young’s modulus $E = 1$ GPa (measured in the linear regime), maximum strength of 28 MPa and maximum strain of 3.25%. b, Optical extinction for a 1 cm sample of carbohydrate glass indicates that the material transmits light wavelengths commonly used during biocompatible imaging and photopolymerization (365–550 nm, shaded box). c, During thermal extrusion and 3D printing, filament diameter is controlled by the travel speed of the extrusion nozzle and follows a simple power law from glass-fibre drawing (equation inset). d, Architectural design of a multiscale carbohydrate-glass lattice (green). e, Top view of the multiscale architectural design in d printed in carbohydrate glass (scale bar, 1 mm). Interfilament melt fusions are magnified and shown in side-view (scale bars, 200 µm). f, Multilayered lattices are fabricated in minutes with precise lateral and axial positioning resolution (scale bar, 1 mm). g, A multiscale architecture showing a single 1 mm filament (top) connected to angled arrays of smaller interconnected filaments (scale bar, 1 mm). h, Serial y-junctions and curved filaments can also be fabricated (scale bars, 1 mm).

varying only the translational velocity of the extrusion nozzle, while holding constant the nozzle diameter and the extrusion flow rate parameters, extruded filament diameters tracked the governing equation:

$$D(v) = \frac{A}{\sqrt{v}}$$

where $D(v)$ is the resultant filament diameter, $A$ is a constant that incorporates the extrusion nozzle diameter and extrusion flow rate, and $v$ is the velocity of the extrusion nozzle (Fig. 1c). This relationship derives from existing models of glass-fibre drawing and allowed the generation of carbohydrate-glass lattices in predefined, multiscale, and reproducible patterns (Fig. 1d–h). Moreover, controlling the temperature of the assembly platform...
Figure 2 | Monolithic tissue construct containing patterned vascular architectures and living cells. a. Schematic overview. An open, interconnected, self-supporting carbohydrate-glass lattice is printed to serve as the sacrificial element for the casting of 3D vascular architectures. The lattice is encapsulated in ECM along with living cells. The lattice is dissolved in minutes in cell media without damage to nearby cells. The process yields a monolithic tissue construct with a vascular architecture that matches the original lattice. b. A single carbohydrate-glass fibre (200 µm in diameter, top) is encapsulated in a fibrin gel. Following ECM crosslinking, the gel and filament are immersed in aqueous solution and the dissolved carbohydrates are flowed out of the resulting channel (middle). Removal of the filament yields an open perfusable channel in the fibrin gel (bottom, scale bar, 500 µm). See Supplementary Movie S1 for full-time course. c. A fibrin gel with patterned interconnected channels of different diameters supports convective and diffusive transport of a fluorescent dextran injected into the channel network (upper left, phase contrast, scale bar, 500 µm). Line plot of normalized fluorescence across the gel and channel (blue arrow) shows a sinusoidal profile in the channel (between dotted black lines) characteristic of a cylinder and temporal diffusion from the channel into the bulk gel. Enlargement of the dotted box region shows an oval intervessel junction between the two adjoining channels. Scale bars, 200 µm. d. Cells constitutively expressing enhanced green fluorescent protein (EGFP) were encapsulated (5 x 10^6 ml^-1) in a variety of ECM materials and then imaged with confocal microscopy to visualize the matrix (red beads), cells (10T1/2, green) and the perfusable vascular lumen (blue beads). They are also shown schematically (bottom right). The materials have varied crosslinking mechanisms (annotated above the images) but were all able to be patterned with vascular channels. Scale bars, 200 µm. e. Representative cross-section image of unlabelled HUVEC (1 x 10^6 ml^-1) and 10T1/2 (1 x 10^6 ml^-1) co-cultures (not expressing EGFP) encapsulated uniformly in the interstitial space of a fibrin gel (10 mg ml^-1) with perfusable networks after two days in culture were stained with a fluorescent live/dead assay (green, Calcein AM; red, Ethidium Homodimer). Cells survive and spread near open cylindrical channels (highlighted with white arrow). Scale bar, 200 µm.

Facilitated the formation of smooth melt fusions at filament intersections (Fig. 1e).

We next sought to use these lattices as a sacrificial element for creating fluidic channels within monolithic cellularized tissue constructs (Fig. 2a). In our strategy, a suspension of cells in ECM prepolymer is poured to encapsulate the lattice. After crosslinking the ECM, the glass filaments are dissolved to form vessels while their interfilament fusions become intervessel junctions (Fig. 2b,c). To prevent disruption of ECM crosslinking and to avoid the potential for osmotic damage to encapsulated cells due to carbohydrate dissolution, we coated the carbohydrate-glass lattice with a thin layer of poly(n-lactide-co-glycolide) (PDLGA) before casting the ECM. This coating allowed the dissolved carbohydrates to be flowed out of the formed channels instead of through the bulk of the engineered construct (Fig. 2b and Supplementary Movie S1). Importantly, the coating did not inhibit the ability of the network to support convective and diffusive transport into the bulk gel (Fig. 2c). Furthermore, we observed that, after sacrifice, the glass interfilament fusions left behind smooth elliptical intervessel junctions that supported fluidic connection between adjoining vascular channels.

To demonstrate the flexibility and generality of this approach, we patterned vascular channels in the presence of living cells in a wide range of natural and synthetic ECM materials (Fig. 2d). The time required for encapsulating cells and lattices in ECM prepolymer, ECM crosslinking, and glass dissolution is on the order of minutes. Importantly, we chose ECM materials which varied not only in their bulk material properties but also in their means of crosslinking. Indeed, the approach generated channels without the need to modify handling of aqueous cellularized gels formed by chain entanglements (cooling of agarose), ionic interactions (calcium-polymerized alginate), photopolymerization (synthetic...
poly(ethylene glycol) (PEG)-based hydrogels\textsuperscript{18}, enzymatic activity (thrombin-polymerized fibrin), and protein precipitation (warming of Matrigel). As predicted from the characterization of the optical transparency of the carbohydrate glass (Fig. 1b), photopolymerized gels exhibited no visible shadowing artefacts due to light absorption by the patterned glass lattice. To our knowledge, no other channel-forming technique is compatible with such a wide range of ECM materials. The approach also seems to have no negative effects on cells. Encapsulated cells survived, spread, and migrated in channelled scaffolds at levels not different from non-channelled control gels, demonstrating biocompatibility of the entire vessel casting process (Fig. 2c). Similar viability was found for human umbilical vein endothelial cells (HUVECs), 10T1/2 cells, human fibroblasts, and human embryonic kidney (HEK) cells (data not shown).

Owing to the mechanical rigidity and self-supporting nature of the carbohydrate-glass lattice, introducing a 3D multilayer architecture into the engineered vasculature requires no additional constraints, time, or steps to the sacrificial process (Supplementary Fig. S1a). To demonstrate the compatibility of this vascular casting approach with additional design considerations often important to the engineering of tissues, we fabricated complex cellular and immobilized-factor gradients in tissue constructs that also contained our channels (Supplementary Fig. S1b). Analysis of these tissue constructs (Supplementary Fig. S1c) demonstrated that patterning of cells or immobilized factors within the construct into step, linear, and exponential gradients could be accomplished in a single engineered tissue construct containing perfusable vascular channels. Here, cells were encapsulated along with immobilized fluorescent beads as a model factor, but this technology should be readily translated to immobilized gradients of adhesive peptides\textsuperscript{19}, proteins and growth factors\textsuperscript{20-22}, or the ECM itself\textsuperscript{23,24}. Together, these results illustrate that the available design parameter space for a tissue construct is, for the first time, unhindered by the inclusion of patterned vascular channels and junctions.

Vascularized solid tissues can be conceptually reduced to a ‘vascular unit cell’ consisting of three key compartments: the vascular lumen, which serves as both the source and sink for most soluble factors; endothelial cells lining the vascular wall, which regulate mass-transport exchange with the interstitium; and cells and matrix residing in the interstitial zone between vascular channels (Fig. 3a). Here, we demonstrate control over each of these compartments in engineered tissue constructs. The monolithic nature of the gels (resulting from a single step polymerization) and the lack of architectural seams supported non-leaking perfusion of human blood under positive pressure with either laminar or turbulent pulsatile flow (Fig. 3b and Supplementary Movies S2 and S3), with smooth interchannel junctions supporting branched fluid flow. Endothelial cells seeded through a single inlet in the network quickly lined the walls of the entire network, including the junctions between vessels of differing diameters (Fig. 3c,d). Because this endothelium is formed after forming the tissue, these cells and their seeding are independently introduced from cells encapsulated in the interstitial zone. In co-cultures with 10T1/2 cells in the interstitial space, endothelial cells lining the vascular lumen became surrounded by the 10T1/2 cells and formed single and multicellular sprouts extending from the patterned vasculature into the bulk gel (Fig. 3e,f).

Engineered constructs densely populated with cells can develop a necrotic core owing to lack of adequate mass transport\textsuperscript{1}. Thus, a major functional requirement of such an engineered vasculature is its utility to sustain cellular activity in metabolically demanding settings such as physiologically high cell densities. To facilitate imaging of such a densely populated construct, we generated a single layer of parallel fluidic channels formed within the centre of a rectangular construct. As a measure of cellular function and activity, we examined expression of destabilized enhanced green fluorescent protein (dsEGFP) from a constitutively expressed lentiviral cassette inserted into HEK293T cells. Monolithic slab gels uniformly distributed with cells exhibited cellular activity only at the gel perimeter (Fig. 4a). In contrast, gels with channels rescued protein expression in the gel core, most dramatically around each perfused channel. This preservation of cell function was perfusion-dependent, as dsEGFP expression was absent near channels without perfusion (data not shown). We also looked at a broader range of cellular densities in these constructs with a functional enzyme assay of a constitutively secreted Gaussia luciferase reporter. At low cell densities, diffusion alone is able to maintain cellular function in bulk gels (Fig. 4b). As nutrient requirements of the tissue construct increased with cell density, Gaussia production began to plateau. This limitation was overcome by convective transport through the channelled scaffold. The maintenance of cellular metabolic activity (Fig. 4a) as well as secretion of functional proteins and enzymes (Fig. 4b) are important functional outputs for many types of engineered tissues\textsuperscript{25}, both of which seem to be maintained with our engineered vasculature. Together these results illustrate the capacity of patterned perfusable channel architectures to provide functional mass transport to 3D cell cultures at or near physiologic cell densities.

Although these data demonstrate the utility of the approach in supporting a transformed cell line, primary parenchymal cells that would ultimately be required for clinically implanted engineered tissues often cannot tolerate stresses associated with extended periods of suspension and hypoxia. We therefore engineered perfusable gels containing primary hepatocytes, which are known to be highly sensitive to hypoxia and handling (Fig. 4c,d). After eight days in culture, perfusable tissues exhibited substantially higher albumin secretion and urea synthesis than slab gels (gels without channels) of the same volume (Fig. 4d). Optical sections of these constructs showed that at high cell concentrations cell survival was most prevalent adjacent to perfused channels and decayed radially, consistent with patterns observed in the dsEGFP-expressing HEK293T cells as well as with a 3D finite-element model of nutrient delivery to the entrapped cells (Supplementary Fig. S2). Together these results illustrate the utility of this strategy for supporting the function of engineered tissues comprising even highly sensitive primary cells.

Existing methods to create cell-laden gels containing a microfluidic network have required the delicate process of precise stacking and lamination of individually fabricated layers\textsuperscript{6-7}. In this study, we found sacrificial carbohydrate-glass lattices to be well suited for the creation of densely populated tissue constructs with perfusable vascular channels and junctions. A key advantage of our method is that the entire perfusable scaffold is formed as a continuous phase simply by filling the 3D void volume around carbohydrate-glass lattices and by crosslinking the matrix. Cells are encapsulated in ECM and the resulting tissue construct can be perfused within minutes. We believe the sheer rapidity of this process prevents the formation of a necrotic core for metabolically demanding cells during fabrication. Moreover, the microstructural characteristics of the fluidic network—such as vessel diameter, circularity, surface roughness, and junction architecture—arise from fibre drawing and surface tension rather than photolithography or micromachining. Relying on simple physical principles, rather than on engineering processes, enabled the use of low-precision hardware to rapidly and reproducibly generate multiscale microvascular architectures in aqueous-based biomaterial scaffolds containing living cells.

Further, the process separation between the 3D microfabrication of filament networks and the handling of cells and ECM allows the dissemination of the technology to distant research laboratories. To illustrate this feature in the current study, carbohydrate-glass lattices were shipped under ambient conditions between laboratories. Primary liver hepatocytes were then encapsulated via standard manual pipetting steps to rapidly create perfusable hepatic
Figure 3 | Demonstrated control over the three key compartments of vascularized solid tissues. a, Schematic of these three compartments in a 'vascular unit cell' consisting of the vascular lumen, endothelial cells lining the vascular wall, and the interstitial zone containing matrix and encapsulated cells. Patterned vascular channels support positive pressure and pulsatile flow of human blood with intervessel junctions supporting branched fluid flow (left). Spiral flow patterns (right, 0.4 s) are characteristic of non-laminar flow through cylindrical channels. See Supplementary Movies S2 and S3; Scale bars, 1 mm, left; 2 mm, right. c, Control of the interstitial zone and the lining endothelium of vascularized tissue constructs is demonstrated by encapsulating 10T1/2 cells (1.5 \times 10^6 \text{ ml}^{-1}, \text{constitutively expressing EGFP}) in the interstitial space of a fibrin gel (10 mg ml\(^{-1}\)) followed by seeding of HUVECs (constitutively expressing mCherry) throughout the vascular network via a single lumenal injection (see Methods). After one day in culture a confocal z-stack montage demonstrated HUVECs residing in the vascular space with 10T1/2 uniformly distributed throughout the bulk gel. Scale bar, 1 mm. d, A partial z-stack of two intersecting channels demonstrated endothelialization of channel walls and across the intervessel junction, while in the surrounding bulk gel 10T1/2 cells are seen beginning to spread out in three dimensions. See Supplementary Movie S4 for the complete 700 \mu m z-stack from d, e. After nine days in culture, cross-section imaging of a representative channel (optical thickness and z-position = 10 \mu m) demonstrated that the endothelial monolayer lining the vascular lumen became surrounded by 10T1/2 cells. Scale bar, 200 \mu m. f, Endothelial cells formed single and multicellular sprouts (arrowheads) from patterned vasculature, as seen in a z-stack (optical thickness = 200 \mu m) from deeper within the gel (z-position = 300 \mu m, left). Even deeper imaging (z-position = 950 \mu m, optical thickness = 100 \mu m, right) confirmed that the vascular lumen remained open throughout vessels and intervessel junctions and that endothelial cells also sprouted from larger vessels (arrowheads). See Supplementary Movie S5 for the complete 1 mm z-stack from e, f.

tissues or non-perfusable control gels. The ability to access this vascularization strategy without fabricating the networks in-house may facilitate rapid adoption of the technology.

Engineered 3D constructs have gained increased attention as in vitro tools for the study of cell–cell and cell–matrix interactions, and are being explored for potential use as experimental models...
or therapeutic replacements of human tissues. However, with the exception of avascular or thin tissues, it has been difficult to achieve the cellular densities of native tissues (approximately 10–500 million cells ml$^{-1}$). The approach described here demonstrates an avenue for building and studying such tissue mimics, in which the vasculature seems not to constrain the design space for the tissue itself, allowing for arbitrary cell types, matrices, and their patterning. Coupled with advances in microfluidic device technologies\textsuperscript{26,27}, the controlled architecture of these engineered fluidic networks may also provide a means to directly examine the interplay between mass transport requirements of specific tissues and vascular architecture. Moreover, the facile and highly automated nature of this perfusable tissue fabrication strategy should provide a flexible platform for a wide array of specific applications, and may enable the scaling of densely populated tissue constructs to arbitrary size.

**Methods**

**Preparation and 3D printing of carbohydrate glass.** A mixture of 25 g glucose, 53 g sucrose, 10 g dextran (86kDa), and 30 ml reverse osmosis water (\(\geq 18 \text{ M}\Omega;\) Millipore) was warmed to 165 °C to remove most of the water and form a liquid glass. The hot mixture was poured into a 30 ml syringe that was maintained at 110 °C. The syringe was mounted on a custom-modified RepRap Mendel 3D printer with associated electronics (Gen3, MakerBot; RAMPS + RAMMS, Ultimachine). Custom Python scripts were developed to generate the 3D motion control GCode used to drive the machine via open-source ReplicatorG software. Carbohydrate-glass lattices were printed at 110 °C under nitrogen pressure with pneumatic control through a 16-gauge or 18-gauge (1.2 mm or 0.84 mm ID, respectively) steel nozzle, vitrified to 50 °C, and then immersed in a 25 mg ml$^{-1}$ solution of PDLGA (Parac) in chloroform for up to 5 min. Glass lattices were encapsulated in ECM along with living cells on the same day the lattices were fabricated, or were stored at 45 °C or in a vacuum chamber until use to protect the hygroscopic carbohydrates from absorbing ambient moisture under atmospheric conditions.

**Polymer synthesis and cell and lattice encapsulation.** Poly(ethylene glycol) diacrylate (PEGDA, 6 or 35 kDa) and acrylate-PEG-RGDS (4 kDa) were synthesized as previously described\textsuperscript{18}. In a typical experiment, a prepolymer mixture containing PEGDA (5, 10 or 20 wt%), acrylate-PEG-RGDS (1 mm), photoinitiator (Lepadure 2959, Ciba Geigy, 0.05% w/v), and cells of interest (1–40 x 10^6 cells ml$^{-1}$) was dispensed into rectangular moulds containing suspended carbohydrate-glass lattices (500 µl total volume per gel). PEG hydrogels were photopolymerized (Omnicure 2000, 320–500 nm) at 100 mW cm$^{-2}$ for two repetitions of 30 s duration (rotating 180° about the y axis before the second exposure). Fibrin gels (10–40 mg ml$^{-1}$) were created by combining fibrinogen, thrombin and cell suspension in phosphate-buffered saline (PBS) and then dispensing this mixture around a carbohydrate-glass lattice. Fibrin gels were polymerized for 10 min at 37 °C or for 20 min at room temperature. Matrigel constructs were formed by mixing a cell suspension with Matrigel and then dispensing the mixture around a carbohydrate-glass lattice, followed by incubation at 37 °C for 10 min. Alginate gels (2%) were formed by mixing an alginate solution with cell suspension, dispensing the mixture around a carbohydrate-glass lattice, then carefully crosslinked with a 90 mg ml$^{-1}$ CaCl$_2$ solution for 10 min. Agarose gels (2%) were formed by mixing a low-melt agarose solution with cell suspension, dispensing the mixture around a carbohydrate-glass lattice, then placed at 4 °C for 20 min. All crosslinked gels were post-processed identically: after crosslinking they were placed in complete medium to dissolve the carbohydrate glass (10 min), followed by exchange with fresh medium and cell culture (static culture or orbital shaking at a rate of 2 Hz). In 800 µm channels (diameter) we measured peak flow.
rates of 10 μl−1, corresponding to mean velocities of 5 mm s−1 and shear stress of 1 dyn cm−2. These values are comparable to physiologic settings. Gel slabs (without vascular architectures) were created by using identical rectangular moulds and identical gel processing steps, but without encapsulating glass lattices. Diffusion studies were conducted with fluorescent Cascade Blue Dextran 10 kDa (Invitrogen). Fluorescent beads (Polysciences) were mixed with ECM prepolymer mixtures for matrix immobilization or PBS for bead perfusion studies. Heparanized human blood, whole blood, or packed red blood cells (Interstate Blood Bank) were washed and diluted with PBS before use.

Endothelialization of vascular networks. HUVECs were seeded in the vascular lumen by injecting a HUVEC suspension (35 × 106 cells ml−1) into the vascular architecture approximately 10 min after the constructs were fabricated. HUVECs were allowed to attach in static culture for one hour before introducing flow, and they reached confluence within one day. Endothelialized gels often contained additional HUVECs (1.5 × 106 ml−1) and 10T1/2 cells (1.5 × 106 ml−1) encapsulated in the bulk gel, such as the gel shown in Fig. 3c–f.

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References


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Author contributions


Additional information

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Omnidirectional Printing of 3D Microvascular Networks

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Nature is replete with examples of microvascular systems that enable efficient fluid flow and distribution for autonomic healing, cooling, and energy harvesting. Emulating these systems in functional materials is of considerable interest for emerging applications in self-healing,[1–2] tissue engineering,[3–5] organ printing,[6,7] microfluidics,[8,9] and biomedical devices.[10] In one example, skin-like mimics containing synthetic microvascular networks filled with healing agents demonstrated repeated repair of damage in a single location.[11]

In another example, tissue engineering constructs containing both embedded cells and a planar array of microchannels were developed to facilitate the delivery of nutrient-laden fluids that promote cell viability.[12–14] Despite these recent examples, the fabrication of synthetic microvascular networks composed of complex, hierarchical 3D architectures remains an elusive goal. Although several techniques, including soft lithography,[14,18] stereolithography,[19] direct laser ablation,[20] bioprinting,[21] and electrostatic discharge[22] have been developed, none combine the scalability, resolution, and repeatability required to synthetically replicate 3D biomimetic microvascular constructs incorporating hierarchical, bifurcating features.

Direct ink writing (DIW) provides an attractive method for creating 3D microvascular structures due to its benign processing conditions and ease of fabrication.[1,3,10,13] In this method, a fugitive organic ink is patterned into the desired motif, encapsulated in a thermally or photocurable resin, and subsequently removed by liquefaction to yield a microvascular network consisting of uniform microchannels interconnected in three dimensions. As originally implemented, its layer-by-layer build sequence limits network designs to simple architectures, such as those based on 3D periodic lattices. Recently, Hansen et al.[24] used dual fugitive inks in combination with vertical printing to create 3D interpenetrating microvascular networks. While these advances permit increased pattern complexity, even this modified approach is unable to create true 3D biomimetic microvascular networks.

Here, we demonstrate the omnidirectional printing (ODP) of 3D biomimetic microvascular networks (Figure 1); a new variant of direct-write assemblies that vastly broadens the network design space by obviating the need for layerwise patterning. In this approach, fugitive ink filaments are printed within a photo-curable gel reservoir, which physically supports the patterned features thereby allowing truly omnidirectional freeform fabrication (Figure 1a). As the deposition nozzle translates through the reservoir, void space is generated locally and filled by the migration of liquid from the fluid capping layer (Figure 1b). The liquid filler is designed to have identical chemical functionality, yet a significantly lower viscosity than the photopolymerizable reservoir. Thus, any voids produced during printing are immediately filled. After printing is completed, the gel reservoir and fluid filler are solidified via photo-polymerization to form a mechanically robust, chemically crosslinked matrix (Figure 1c).

Because the fugitive ink has not been chemically modified, it can be subsequently removed by liquefaction at 4 °C under a modest vacuum to yield the desired microchannel network within the matrix (Figure 1d,e).

To enable ODP of 3D biomimetic microvascular networks, we developed a chemically compatible fugitive ink, fluid filler, and physical gel reservoir, whose properties are tailored for printing and subsequent transformation into the desired structures. Specifically, we created a fluorescently dyed, fugitive ink composed of an aqueous solution of Pluronic F127, a triblock copolymer with a hydrophobic poly(propylene oxide) (PPO) segment and two hydrophilic poly(ethylene oxide) (PEO) segments arranged in a PEO–PPO–PEO configuration and a diacrylate-functionalized Pluronic F127 solution[25–27] of varying concentration as the fluid filler and physical gel reservoir, respectively.[28] Using this system, we constructed 3D biomimetic microvascular networks composed of a hierarchical, 3-generation branching topology with microchannel diameters ranging from 200–600 μm, in which two large parent channels are subdivided into many smaller microchannels (Figure 1f).

Aqueous Pluronic F127 triblock copolymer solutions undergo a phase transition that is both concentration and temperature dependent.[29–31] Under ambient conditions, the PEO–PPO–PEO species form micelles that consist of a PPO core surrounded by a PEO corona above a critical micelle concentration (CMC) of ~21 w/w% yielding a physical gel, as reflected by the substantial increase in shear elastic modulus, G′ as the concentration exceeds the CMC (Figure 2a). As reported previously, these spherical micelles have an average hydrodynamic diameter ranging from 20–80 nm.[29] In addition, these Pluronic F127 solutions also possess a critical micelle temperature (CMT) of ~10 °C (Figure 2b). Above the CMT, the PPO block dehydrates leading to pronounced hydrophobic interactions that drive micelle formation. However, below the CMT, the hydrophilic PPO units are hydrated, allowing individual PEO–PPO–PEO species to become soluble in water thereby inducing a gel-to-fluid transition for systems, whose concentration resides above the CMC. Concomitantly, there is an abrupt decrease in G′ at temperatures below ~10 °C.

We exploit the known phase behavior of aqueous Pluronic F127 solutions in the design of our ink, matrix, and fluid filler. Our fugitive ink consists of an aqueous solution of Pluronic F127 that resides slightly above the CMC. This ink exhibits pronounced shear thinning behavior (Figure S1, Supporting Information) and shear elastic plateau modulus G′ that exceeds

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enough to allow ink removal. To meet this latter criteria, we chemically modified the terminal hydroxyl groups of the PEO segments with diacrylate groups.[29–31] In its chemically modified, uncured state, Pluronic F127 diacrylate (F127-DA) solutions exhibit a CMC of 23 w/w% (Figure 2c), which is slightly higher than their non-functionalized counterpart. We used a F127-DA gel that possesses a viscosity and \( G' \) similar to that of the pure fugitive ink (Figure S1, Supporting Information). Importantly, this reservoir can be photopolymerized to produce a chemically cross-linked matrix by incorporating a photoinitiator followed by irradiation of 365 nm UV light for 5 min.

During ODP, the nozzle translates through the uncured Pluronic F127-DA gel to deposit fugitive ink in the desired 3D microvascular network pattern. As this process occurs, void spaces are introduced into the matrix. To fill these crevasses, we used a fluid filler composed of a liquid solution of the acrylate-modified Pluronic F127, which is placed on top of the matrix as a capping layer (1 mm thick). Because its concentration lies below the CMC, the fluid filler exhibits Newtownian behavior under ambient conditions with a viscosity of \( 6.6 \times 10^{-1} \) Pa s and a negligible shear elastic modulus (Figure 2c). However, since the filler fluid is chemically identical to the supporting reservoir, it can be photocured after patterning of the 3D microvascular network is complete.

Next, we investigated the printing behavior of the fugitive ink, hydrogel reservoir, and fluid filler over a range of compositions to determine their respective optimal formulations for ODP. Our assessment of ink performance included the ability to cleanly start and stop ink flow, to control the microchannel diameter by dynamic pressure variation, and to retain its filametary shape during and after deposition. We also assessed the reservoir and fluid filler performance; the reservoir must support the deposited ink filaments without disruption due to viscous drag as the nozzle translates through nearby regions, while the fluid must rapidly fill gaps formed within the reservoir during the patterning process. Optimal concentrations of \( \sim 23 \) w/w% for the ink and 25 w/w% reservoir were determined for ODP (Figure 2d), both of which reside above their respective critical micelle concentrations of 21 w/w% and 23 w/w% (Figure 2a,b). Fluid inks exhibited drop-breakup leading to the formation of discontinuous filaments. For this ink/reservoir combination, a minimum ink elastic modulus of \( >10^4 \) Pa is required for filamentary printing. To control the microchannel diameter, we employed a dynamic pressure variation approach in which a single nozzle

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**Figure 1.** a–e) Schematics of omnidirectional printing of 3D microvascular networks within a hydrogel reservoir. a) Deposition of a fugitive ink into a physical gel reservoir allows hierarchical, branching networks to be patterned. b) Voids induced by nozzle translation are filled with liquid that migrates from the fluid capping layer. c) Subsequent photopolymerization of the reservoir yields a chemically cross-linked, hydrogel matrix. d,e) The ink is liquefied and removed under a modest vacuum to expose the microvascular channels. f) Fluorescent image of a 3D microvascular network fabricated via omnidirectional printing of a fugitive ink (dyed red) within a photopolymerized Pluronic F127-diacrylate matrix. (scale bar = 10 mm)
is used to pattern microchannels of varying size. When the applied pressure ($P$) and writing speed ($v$) are controlled such that the volumetric flow rate ($Q$) equals $0.25\pi D_{\text{micro}}^2 v$, the microchannel diameter ($D_{\text{channel}}$) is nearly equal to the nozzle diameter ($D_{\text{nozzle}}$). However, this approach would require the use of multiple nozzles to pattern hierarchical, bifurcating microchannels.\(^{23}\) To overcome this limitation, we varied the applied pressure at a constant writing speed. We observed a linear increase in the microchannel diameter with applied pressure for fugitive ink deposition into the hydrogel reservoir (Figure 3a). While this trend is consistent, we found that the slope varied with nozzle diameter. For example, microchannels with diameters ranging from 18 μm to 170 μm ($D_{\text{channel}}$ = 0.5 to 3.5$D_{\text{nozzle}}$, respectively) could be achieved using a 30 μm tapered glass capillary (Figure 3b,c), which is the smallest nozzle through which this ink readily flows. Although features slightly smaller than the nozzle diameter were obtained, further reductions in the applied pressure led to filament breakup. Microchannels of up to 600 μm ($D_{\text{channel}} = 3D_{\text{nozzle}}$) could be formed using a 200 μm cylindrical stainless steel nozzle. However, this does not constitute an upper bound, since larger microchannels (>1 mm) can be produced simply by using a large nozzle size (see Supplementary Movie 1, Supporting Information).

To examine the effect of initial reservoir and filler fluid composition on the final mechanical properties of the cured Pluronic F127-DA matrix, we performed a dynamical analysis of 1" circular specimens to obtain their elastic modulus $E'$ as a function of concentration. Unlike the strong concentration dependence observed for $G'$, the mechanical properties ($E'$) of the cured system depend only modestly on concentration (Figure 3d). This finding indicates that $E'$ is influenced primarily by the formation of a chemically crosslinked matrix rather than its initial physical gel or fluid state. Hence, the incorporation of fluid filler within the reservoir during omnidirectional printing does not adversely affect the final mechanical properties of the bulk matrix.

Hydrogel matrices with embedded 3D biomimetic microvascular networks may find potential application in tissue engineering\(^{32}\) and drug delivery.\(^{19}\) In these applications, the diffusion rate of solutes from fluid-filled microchannels into the surrounding matrix imposes additional design constraints on the microvascular network. For example, in cell-seeding applications,\(^{14–17}\) nutrient diffusion is limited to several hundred microns,\(^{34}\) which dictates in part the maximum allowable microchannel spacing. To characterize the diffusivity ($D$) of a low molecular weight solute within the cross-linked Pluronic F127-DA matrix, we injected a rhodamine-based fluorescent dye into a 125 μm microchannel and allowed it to diffuse into the matrix under quiescent conditions (Figure 4a). The diffusion profile acquired by fluorescent imaging consists of averaged intensities normalized to $r^2 = 0$ (Figure 4b) and shows the progression of the dye through the matrix. For the image analysis, the intensity is assumed to be proportional to the concentration. The spatial peak variance, $\sigma^2$, is extracted from 1D Gaussian fits from each profile with $R^2 > 0.98$, excluding the channel region. Using the Einstein-Smoluchowski relation, the diffusion constant is determined from the plot of $\sigma^2$ as a function of time (Figure 4c). Specifically, we found a diffusion constant $D = 2.15 \times 10^{-7} \text{cm}^2 \text{s}^{-1}$ for a 0.1 w/w% dye in a 25 w/w% Pluronic F127-DA matrix. As a control, we verified the validity of this approach using an agar system, where the measured diffusivity was consistent with reported values (see Supporting Information).

In summary, we have fabricated 3D biomimetic microvascular networks embedded within a hydrogel matrix via omnidirectional printing. This novel approach hinges critically on tailoring the chemical and rheological properties of the fugitive ink as well as the photopolymerizable hydrogel reservoir and fluid filler. These hydrogel-based, microvascular constructs may find potential application in 3D cell culture, tissue
engineering, organ modeling, or autonomic healing. Looking ahead, this approach may be readily extended to create complex 3D composites by incorporating nanoparticles or other functional building blocks within the ink, reservoir, or both components of the system.

Experimental Section

Preparation of the Pluronic F127 Fugitive Ink: The fugitive ink is produced by dissolving Pluronic F127 (Sigma-Aldrich) into 4 °C deionized water at 23 w/w% and stirring vigorously for 3 h. Several drops of a water-soluble fluorescent dye are added to the ink to aid in visualization during omnidirectional printing. The solution is stored overnight in a refrigerator to allow air bubbles to dissipate. The cooled ink is poured into 5 mL syringes (EFD-Inc) and warmed to room temperature prior to printing.

Synthesis of Pluronic F127-DA: 100 g of Pluronic F127 (Sigma-Aldrich) is dissolved into 400 ml of dry toluene at 60 °C in a oven-dried three-neck round-bottom flask fitted with a condenser with circulating cold (20 °C) water to minimize solvent evaporation. The solution is allowed to cool to room temperature using an ice bath under a dry nitrogen flow. Triethylamine (Sigma-Aldrich) is added to the solution at a molar ratio of 10:1 Pluronic F127-DA while stirring. Acryloyl chloride (Sigma-Aldrich) at a molar ratio of 10:1 Pluronic F127-DA is added to the flask drop-wise, and the reaction is allowed to proceed while stirring overnight at 25 °C under nitrogen. The yellow triethylammonium hydrochloride is filtered and the remaining Pluronic F127-DA solution is precipitated in a 1:1 ratio of hexane. The final product is filtered and dried at room temperature for 24 h.

Preparation of Pluronic F127-DA Fluid Filler and Matrix: The Pluronic F127-DA fluid filler and matrix is prepared at 20 and 25 w/w% respectively by mixing in cold water and stirring vigorously for 3 hrs. 1 w/w% of a photoinitiator (Dargocure 1173, Ciba) is added, and the solution is then allowed to defoam in a refrigerator overnight.

Omnidirectional Printing: 3D microvascular branched networks are fabricated using a three-axis robotic deposition stage (ABL5000, Aerotech Inc.) by printing a 3D pattern into a physical gel reservoir. The 3D patterns are designed using commercially available CAD software (AutoCAD 2010, Autodesk) and translated into G-code instructions for the deposition stage using a custom Visual Basic program. The 23 w/w% Pluronic F127 ink is housed in a 5 mL syringe fitted with nozzles ranging in size from 10 μm to 200 μm (World Precision Instruments and Nordson EFD). Prior to deposition, a reservoir of 25 w/w% Pluronic F127-DA is poured into a silicone mold at 4 °C and slowly solidified by warming to room temperature, inducing the formation of micelles. The nozzle tip is inserted into the bottom of the gel reservoir and a 1 mm thick layer of a 20 w/w% Pluronic F127-DA filler fluid is poured on top of the gel reservoir prior to printing. Air pressure (Ultimus V, Nordson EFD) is applied to initiate extrusion. The ink filament size is varied dynamically, by first printing calibration standards that consist of arrays of parallel channels at various pressures and measuring their printed dimensions by optical microscopy. From these data, the linear relationship between applied pressure and filament size was determined. After deposition, the Pluronic F127-DA is chemically cross-linked under 365 nm UV light for 5 minutes. The ink is subsequently removed by exposing the largest (parent) channels with a razor blade, cooling the structure under ice water, and applying a light vacuum.

Characterization of Rheology and Mechanical Properties: The rheological properties of aqueous Pluronic F127 and F127-DA solutions of varying concentration are measured using a controlled-stress rheometer (Bohlin CVOR-200, Malvern Instruments) fitted with a C14 cup and bob geometry. 2.5 mL of the solutions are poured into the geometry at 4 °C and allowed to equilibrate to room temperature. Oscillatory-shear measurements are carried out at 1 Hz with a stress amplitude range of 0.1 to 2000 Pa. Viscosity measurements are performed at a shear rate of 0.01 to 100 s⁻¹. Dynamic mechanical
Diffusion of a rhodamine-based dye through photocured Pluronic F127-DA. a) Fluorescent images of microchannels (120 μm in diameter) at \( t^* = 0, 1, 5, \) and 10 min, where \( t^* = 0 \) designates the time that the first image is taken after dye injection. b) Diffusion profiles of the fluorescent intensity normalized to the maximum at \( t^* = 0 \). c) The spatial peak variance \( \sigma^2 \) obtained from Gaussian fits of the intensity profiles plotted as a function of time, from which the slope yields a dye diffusivity of 2.15 \( \times 10^{-7} \text{ cm}^2 \text{ s}^{-1} \).

\[ C(x, t) = \frac{N/A}{\sqrt{4\pi Dt}} \exp \left( -\frac{(x-x_0)^2}{4Dt} \right) \]  

(1)

where \( C \) is the dye concentration, \( N \) is the number of dye molecules, \( A \) is the cross-sectional area through which they diffuse, \( x_0 \) is the position of the line source along the \( x \) axis, perpendicular to the channel length, \( D \) is the diffusion constant, and \( t \) is the time. Using the Einstein-Smoluchowski relation

\[ \sigma^2 = 2Dt \]  

(2)

where \( \sigma^2 \) is the spatial peak variance, the equation can be rewritten as a standard Gaussian function:

\[ C(x, t) = \frac{N/A}{\sqrt{2\pi \sigma^2}} \exp \left( -\frac{(x-x_0)^2}{2\sigma^2} \right) \]  

(3)

At each time step, the fluorescent intensity profile is fitted to a Gaussian function in MATLAB to obtain the fitting parameter \( \sigma^2 \), the spatial peak variance. Shadow regions caused by a mismatch in the index of refraction were omitted from the fit. The diffusion constant is determined from the slope of \( \sigma^2 \) plotted as a function of diffusion time, \( t \).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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