Biofabrication

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Fabrication of microvascular constructs using high resolution electrohydrodynamic inkjet printing

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Abstract

Fabrication of the intricate anatomy of vasculature within engineered tissue remains one of the key challenges facing the field of tissue engineering. We report the use of electrohydrodynamic (EHD) inkjet printing to create hydrogel-based microvascular tissues with hierarchical and branching channels, whose minimum feature size of 30 µm approaches the physical scale of native capillary blood vessels. The principle relies on the use of complementary thermoreversible gelling properties of Pluronic F127 (PF-127) and gelatin methacryloyl, which served as sacrificial templates and permanent matrices respectively. Human dermal fibroblasts and human umbilical vein endothelial cells were successfully co-cultured within the engineered microvascular tissue constructs for up to 21 days, and attained high cell viability. Tissue specific morphology was maintained on perfusion. The ability to create cellularised, vascularised proto-tissues with high spatial resolution using EHD inkjet printing, provides a new strategy for developing advanced vascular models with the potential to impact upon an extensive range of biomedical applications.

1. Introduction

The field of regenerative medicine promises to progress the translation of engineered tissues towards the clinical environment for the benefits of patients [1, 2]. The success of tissue engineered constructs relies on a stable vascular network to facilitate the diffusion of nutrients, oxygen, growth factors and biochemical signalling factors to cells, thereby supporting their various biological functions [3–8]. The diffusion limits of vascular networks are approximately 100–200 µm; hence, distances greater than this lead to ischemic conditions and cell death [9, 10]. To provide sufficient mass exchange, blood vessels with a hierarchy of branches (diameters ranging from 4 to 300 µm) perfuse the tissue down to the capillary scale [11, 12]. The fractal patterning of the capillaries is structurally unique and beyond the scale achievable with current practical bioprinting methods.

Additive manufacturing or three-dimensional (3D) printing methods now have a long history of use as a strategy to build scaffolds or proto-tissues for applications in tissue engineering and regenerative medicine [13–15]. With proven flexibility and versatility, extrusion-based 3D printing has been widely used to build 3D scaffolds with sacrificial inks, integrated with post processing procedures to remove the sacrificial networks and perfuse the resulting channels with endothelial cells (ECs) [16–19]. Limited by the size of the printhead nozzle and rheological properties of the sacrificial inks, extrusion-based 3D printing faces a challenge of improving its resolution to fabricate microvascular networks with diameters <100 µm [15]. Laser-based stereolithography (SLA) is a well-known technique presenting high spatial control. It uses a fast-scanning laser spot-illumination to initiate local photopolymerization within photocurable resins [20, 21]. However, the serial line-by-line writing fashion is time-consuming for creating large-scale objects. Digital light processing (DLP), utilizes millions of digital micromirror arrays to polymerize an imaged plane in a single pass offer printing speeds and comparable resolution with SLA for building complex architectures in a micrometre scale [22, 23]. Living cells can be encapsulated into the 3D constructs using appropriate photo-crosslinkable hydrogels during the DLP fabrication process [24, 25]. However, because both SLA and DLP are based on the photopolymerization of a solution, many biomaterials that cannot be photopolymerized are
excluded from these light-based additive manufacture approaches.

Electrohydrodynamic (EHD) inkjet printing, is a distinct material jetting technique that is related to conventional drop-on-demand inkjet printing but produces droplets considerably smaller in volume [26, 27]. Differing from conventional drop-on-demand inkjet printing, EHD inkjet printing uses electric fields, rather than thermal or acoustic energy, to create the fluid flows necessary for delivering inks to a substrate [28]. By applying an electric potential difference between the printhead nozzle and the substrate, the ink fluid is charged, and the combination of electrostatic and capillary forces leads to the formation of a characteristic conical shape, the Taylor cone [29]. With the use of a pulsed potential, individual drops can be ejected driven by the electric force. This drop formation mechanism allows EHD inkjet printing to achieve droplet volumes in the 1 aL–fL range (10⁻²¹–10⁻¹⁸ m³) and a consequent line resolution from about 100 nm to a few microns (dependant on ink surface contact angle [30]) after printing on substrates. This improves the potential feature resolution compared to that of extrusion-based or conventional inkjet 3D printing by about three orders of magnitude. This high-resolution printing capability has enabled EHD inkjet printing to be used for a range of nano-/micro-fabrication applications, including: DNA microarray printing [31], thin film transistor fabrication [32] and organic light emitting diode devices [33]. The process is highly compatible with the deposition of hydrogels, thus EHD inkjet printing has the potential to enable the fabrication of artificial microvasculature replicas at the native physical scale. Note that although EHD printing has been used to produce features with dimensions <100 nm [34, 35], these are created after removal of a liquid carrier phase, and are therefore somewhat smaller than potential hydrogel structures produced by this method.

Recently, Nie et al reported the fabrication of multiscale vascular chips using a combination of high temperature extrusion printing and melt electrospinning [36]. Polycaprolactone, was deposited into complex patterns with smallest dimension <5 µm, serving as positive channel moulds. Hydrogel-based vascular constructs were created through a ‘casting—peeling-off—alignment—reassemble’ process. With the combination of two types of 3D printing techniques, this study obtained artificial vasculature with a wide range of dimensions (10–500 µm), which is beyond the capability of conventional lithographic methods. However, Nie’s method requires a complicated manufacturing process, especially during the steps of ‘alignment’ and ‘reassemble’ which might influence efficiency and repeatability.

In this work, we present a simple approach based on EHD inkjet printing, coupled with a fugitive material, to create cell-embedded microvascular constructs with a spatial resolution significantly higher than extrusion based bioprinting. Aqueous solution inks of Pluronic F127 (PF-127) were printed to provide sacrificial templates to form artificial microvascular networks with a minimum channel diameter of 30 µm or 60 µm, embedded within a gelatin methacryloyl (GelMA) hydrogel. Human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs) were seeded into the engineered constructs to generate the structural characteristics of a proto-tissue within an extracellular matrix (ECM). These cells formed supportive ECM material and a functional endothelial layer after time in culture, exhibiting basic elements of a bioengineered artificial tissue with capillary scale vasculature. This approach provides a potential platform for creating artificial microvasculature at the capillary scale in 3D.

2. Materials and methods

2.1. Materials synthesis

The synthesis of GelMA was achieved following a sequential pH adjustment method, reported previously [48]. Briefly, 20 g of type A gelatin (175 bloom) derived from porcine skin tissue (Sigma-Aldrich, Gillingham, UK) was dissolved in 200 ml 0.1 M carbonate-bicarbonate buffer (3.18 g Na₂CO₃ and 5.86 g NaHCO₃ in 1 l distilled water), 5 M NaOH or 6 M HCl was added as appropriate to achieve pH = 9.0. Methacrylic anhydride (Sigma-Aldrich) was sequentially added (0.167 ml at each step) to the gelatin solution every 30 min at 50 °C. The solution pH value was monitored and readjusted to 9.0 before each addition. The reaction was continued for 3 h, and then the pH was readjusted to 7.0 to arrest the reaction. The solution was dialyzed against distilled water using 12–14 kDa cut-off dialysis tubing for 1 week at 40 °C, to remove salts and methacrylic acid. The solution was lyophilized for 1 week (held at −80 °C for 24 h in 50 ml tubes and then under vacuum for 6 d), to generate a white porous foam and stored at −20 °C until further use.

The most widely used photoinitiators in cell encapsulation studies using GelMA are hydroxy-1-[4-(2-hydroxyethoxy)phenoxy]-2-methyl-1-propanone (Irgacure 2959) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) because of their proven cytocompatibility [37]. Here we have selected LAP as the photoinitiator of choice due to its better match of adsorption with the 365 nm light source used in this study. The LAP photoinitiator was synthesized in a two-step process as originally described by Majima et al [38]. Dimethyl phenylphosphonite (Sigma-Aldrich) was reacted with 2,4,6-trimethylbenzoyl chloride (Sigma–Aldrich) via a Michaelis–Arbuzov reaction. 1.6 g (0.009 mol) of 2,4,6-trimethylbenzoyl chloride was added dropwise to an equimolar amount of continuously stirred
dimethyl phenylphosphonite (1.5 g) and reacted at room temperature under argon for 18 h. Four-fold excess of lithium bromide (Sigma-Aldrich) in 50 ml of 2-butanone (Sigma-Aldrich) was added to the reaction mixture and heated to 50 °C for 10 min. The mixture was cooled to ambient temperature, allowed to rest for 4 h, and then filtered. The filtrate was washed and filtered three times with 2-butanone to remove unreacted lithium bromide, and excess solvent was dried by vacuum. The final white powder product was recovered in near quantitative yields and analysed by proton nuclear magnetic resonance (1H), matching literature data [37].

2.2. Ink, polymer solution and substrate preparation

An aqueous solution of 10 wt% Pluronic F127 (PF-127, Sigma-Aldrich) in deionized water was used as the fugitive ink. The powder was homogenized in an ice bath with a magnetic stirrer, until fully dissolved. The ink was filtered using a 0.22 µm syringe filter (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently stored at 4 °C until further use.

This study used GelMA as a pure hydrogel for structural fabrication and as a matrix for cell encapsulation. To prepare a pure GelMA solution, GelMA solid foam was dissolved in warm phosphate buffered saline (PBS, Thermo Fisher Scientific) at 15 wt%, with the LAP initiator added at 0.05 wt%. The mixture was vortex mixed and incubated at 37 °C until fully dissolved. The solution was filtered by a 0.22 µm syringe filter and subsequently stored in dark conditions at 4 °C until further use.

For cell encapsulation studies, normal human neonatal dermal fibroblasts (HDFs, PromoCell, Heidelberg, Germany) and green fluorescent protein expressing HDFs (GFP-HDFs, Angio Proteomie, Boston, MA, USA) were used. Cells were first removed from culture flasks via the standard trypsinization procedure, then the cells were dispersed in a solution of GelMA and Dulbecco’s Modified Eagle Medium (DMEM), with 15 wt% GelMA, 0.05 wt% LAP, at a concentration of 2 × 10⁶ cells ml⁻¹ and gently pipetted up and down to mix evenly. A new batch of GelMA cell suspension was freshly prepared just prior to use.

To prepare polydimethylsiloxane (PDMS) substrates with flat surfaces, degassed PDMS (Sylgard 184, Dow Corning, Midland, MA, USA) liquid mixture (10:1, oligomer:hardener) was poured onto a silicon wafer (diameter 76.2 mm, Agar Scientific, Stansted, UK), contained in a Petri dish (100 × 15 mm). Thickness was set at 2 mm and the mixture cured at 70 °C for 2 h. The PDMS was then peeled from the silicon wafer and trimmed into 20 × 20 mm squares. To improve cell adhesion, the surface of PDMS squares was coated by polydopamine (PDA). The coating treatment followed a protocol described previously [39] with minor modifications. Briefly, dopamine hydrochloride (Sigma-Aldrich) at 2 mg ml⁻¹ (10.6 mM) was dissolved in sodium acetate (Sigma-Aldrich) buffer at 50 mM, pH = 5.0. Next, sodium periodate (Sigma-Aldrich) was added as the oxidant to the solution at 4.5 mg ml⁻¹ (21.2 mM) and quickly vortex mixed until fully dissolved. The PDMS squares were then immediately immersed in the solution and incubated at 40 °C for 2 h. After the coating process, the squares were rinsed with deionized water, air-dried and stored in dark conditions until further use.

2.3. Construct fabrication

Bioprinting was carried out using a commercial EHD inkjet printing system (SLT5050-KBD, Sij Technology, Tsukuba, Japan). This was equipped with a glass capillary nozzle (tip diameter 2–2.5 µm), which was filled with 20 µl of the PF-127 ink using a micropipette. The ink was deposited onto a PDA-coated PDMS square substrate by applying pulsed voltage >300 V. For monolayer printing, the nozzle tip ran at a working distance of ~3 µm from the substrate, while for multilayer printing, the nozzle tip was raised by 1 µm every three layers.

Either pure GelMA solution or the GelMA cell suspension was warmed to 37 °C and cast onto the Pluronic patterned PDA-coated PDMS substrates, which were contained in a 20 × 20 mm acrylic plastic square box. The casting thickness was controlled at ~500 µm. Following this, the GelMA hydrogel was crosslinked by illuminating with a UV light source (365 nm, 5 mW cm⁻²) for 30 s. After crosslinking, the entire construct was flipped over and two stainless-steel blunt nozzles with diameter 1 mm were inserted through the PDMS substrate at two positions of inlet and outlet. Nozzles were connected with an external tubing (0.86 mm LDPE tubing, Portex, Smith Medical, UK). Finally, the constructs were cooled to 4 °C to liquify the embedded PF-127 features which were extracted by applying a modest vacuum through the external tubing. The exposed hollow microchannels were rinsed with PBS buffer for three times, prior to further work of cell seeding and maintenance in culture. Control specimens were prepared using identical substrates, without the printed Pluronic pattern, using the same volume of cell suspension and hence cast thickness.

2.4. Cell seeding and culture

Both normal human neonatal dermal fibroblasts (HDFs, PromoCell, Heidelberg, Germany) and GFP-HDFs (Angio Proteomie, Boston, MA, USA) were maintained in DMEM containing high glucose and sodium pyruvate (DMEM, GlutaMAXTM, Gibco—Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Both primary HUVECs (PromoCell, Germany)
and red fluorescent protein expressing HUVECs (RFP-HUVECs, Angio Proteomic, USA) were maintained in EGM-2 medium (PromoCell, Germany) containing 1% penicillin/streptomycin (Thermo Fisher Scientific). All the cell cultures were passaged following the respective vendor’s instructions and incubated at 37 °C, 5% CO₂. No cells were used beyond the ninth passage.

Prior to HUVEC seeding, the internal surfaces of the microchannels were coated with fibronectin to improve cell adhesion. Fibronectin solution at 0.01 mg ml⁻¹ (Sigma-Aldrich) was injected into the microchannels and incubated at 37 °C for 30 min. Then, the microvascular networks were flushed with fresh EGM-2 medium. 20 µl of HUVECs in suspension at the concentration of 1 × 10⁷ cells ml⁻¹ was injected to fill the network. Prior to the injection, the cell suspension had to be evenly mixed to prevent the formation of cell clumps which might block the microchannels during injection. The external tubing was then sealed, and the perfused construct was incubated at 37 °C under static conditions. After 60 min, the construct was flipped over to facilitate HUVEC adherence to the other side of the microchannels. After 2 h, non-adherent cells were flushed out with fresh EGM-2 medium. The HUVEC seeded constructs were incubated at 37 °C and maintained by changing the medium every 12 h. To avoid HUVECs detaching from the surface of microchannels, it was found that the flow rate within the channels must be <2 µl min⁻¹.

2.5. Cell and construct assessment

Cell viability within the gels was assessed by live/dead staining. Prior to staining, the cell culture medium was removed from cell-containing hydrogel constructs and washed with PBS buffer three times. The constructs were immersed in the staining solution of calcein-AM (‘live’; 1 µl ml⁻¹, Invitrogen) and ethidium homodimer (‘dead’; 4 µl ml⁻¹, Invitrogen) incubated at 37 °C for 40 min. In the case of vascularised constructs the solution was also injected into the microchannels. Data were acquired using a confocal microscope (TCS SP5, Leica, Wetzlar, Germany) for each time point (t = 1, 3, 5, 7 d, repeats n = 5), and the mean viability and standard deviations were determined for each sample.

Immunostaining and confocal microscopy were used to image and assess the vascularized proto-tissue constructs. Cell culture medium was first removed from the tissue constructs and they were washed with PBS buffer three times. Next, 10% buffered formalin solution was perfused into the microchannels, and the entire construct was also bathed in the formalin solution. It was fixed at room temperature for 1 h. The fixed construct was then perfused with PBS and washed five times. After washing, the construct was permeabilized in a solution of 0.2 wt% Triton X-100 in PBS for 1 h. Samples were then stained with 1:2500 DAPI (Sigma-Aldrich) in PBS and 1:20 phalloidin (Invitrogen) in PBS for 4 h. The fluorescence images were captured using a TCS SP5 confocal microscope (Leica) with water-immersion objectives ranging from 10× to 40× using spectral lasers at 405, 488, 561, and 633 nm wavelengths. Z-stack images were processed by the Leica provided software.

Fluorescein isothiocyanate–dextran (FITC-dextran), dye, using 70 kDa dextran, was used to visualize both bare and cell containing constructs. For bare constructs, 25 µg ml⁻¹ of FITC-dextran solution (FITC-dextran, Sigma-Aldrich) was directly perfused into the microchannels. For cell containing constructs, cell culture medium was first removed and washed in PBS buffer three times, 25 µg ml⁻¹ of Far-red-dextran (Alexa Fluor 647, Invitrogen) solution was then injected at a flow rate <2 µl min⁻¹. The fluorescence images were captured using a TCS SP65 confocal microscope and Z-stack images were processed. Far-red-dextran was marked as a blue pseudocolour during image post-processing.

3. Results and discussion

3.1. Fabrication of microvascular tissue constructs

To fabricate the microvascular tissue constructs, PF-127 solution and GelMA hydrogel were applied as the fugitive ink and bulk matrix, respectively. A schematic of the fabrication process is shown in figure 1. Structures with identical microvascular channels were produced using GelMA and a suspension of HDF cells in GelMA. The printing conditions selected in conjunction with 10 wt% Pluronic F127 ink produced individual droplets with a mean volume ≈30 fl (equivalent to a diameter of 3.8 µm), which is approximately three orders of magnitude smaller than achievable with conventional inkjet printing [26]. The small drop volumes lead to rapid gelling of the ink and drying immediately after deposition (estimated as <0.5 s). This leads to a small feature width when printing lines and the absence of defects associated with drying, such as the coffee ring effect (figure 2).

The feature width is controlled by adjusting the spacing of the drops [30] and both width and height can be varied by repeated printing of layers of the ink in the same location. Figure 2(B) shows a scanning electron microscope (SEM) image of a dried Pluronic ridge produced by overprinting 20 layers, with the layered structure evident. Figures 2(C) and (D) show surface profile reconstructions of Pluronic ridges produced after printing 10 and 50 layers respectively. The Pluronic is rehydrated during the GelMA casting process by absorbing water from the GelMA solution after casting at 37 °C. This temperature is higher than the gel point of PF-127 hydrogel, so the rehydrated ridge is maintained as a solid gel phase rather than dissolving into the GelMA solution. To obtain an approximately semicircular profile channel of 60 µm diameter after rehydration, it
Figure 1. Schematic of the manufacturing process using a sacrificial Pluronic F127 solution to form the microvasculature followed by casting a cell-containing GelMA suspension and photopolymerisation.

Figure 2. (A) Image of the EHD inkjet printer in operation showing position of printer nozzle relative to the substrate. (B) SEM image of a printed Pluronic line formed by 20 repeated overprinting operations. Reconstructions of the printed ridges from optical profilometry data after: (C) 10, and (D) 50 layers overprinting.

was necessary to print 100 layers at a drop spacing of 3 μm. By adjusting the number of printed layers and drop spacing, vascular channels with diameters in the range 20–60 μm were printed. For further study with cell containing hydrogels two size scales were printed with minimum channel diameters of 30 μm and 60 μm, respectively. The PF-127 gel served as a temporary support to allow the patterning of internal features during the casting process, and was removed by liquifying at low temperature (4 °C) after the GelMA was photo-crosslinked and gelled.
A typical microvascular network pattern is shown in figure 3(A). It was designed to mimic the hierarchical, bifurcating motifs found in biological systems, in which large channels bifurcate to form smaller channels that maximize efficient blood flow, nutrient transport, and waste removal while minimizing the metabolic cost [9]. The central area of the pattern was designed as a hexagonal network to mimic the arteriole-venule connections at the capillary scale. Figure 3(B) displays an optical image of a microvascular tissue construct printed from the design in figure 3(A). The engineered construct is fabricated on a PDMS substrate, which serves as a waterproof and self-sealing base. The PDMS substrate allows stainless steel needles to be inserted securely through at the inlet and outlet of the vascular mimic, to provide connection with external tubing. This eliminates the needles being inserted directly into the main body of the relatively fragile GelMA hydrogel. The PDMS surface is hydrophobic, which limits the affinity of mammalian cells to effectively adhere [40, 41]. Thus, a PDA coating was deposited on the PDMS, after which the water contact angle decreased from 121.9 ± 1.8° to 31.6 ± 0.8°.

To visualize the microvascular networks, FITCdextran solution was perfused into the microchannels and laser confocal microscopy was used to image the cross-sectional lumen profile via Z-stack projection. Figure 4 shows the central region of the network, in which larger channels bifurcate to two smaller channels, and their smallest branches connect each other to form a hexagonal network; here, the smallest channels were fabricated with the diameters of 30 µm and 60 µm, respectively. The images of the Z-stack 3D projections show a dome-like cross-section. Unlike soft lithography, which creates rectangular channels with right angles, these half cylindrical channels with rounded corners fabricated by high resolution inkjet printing provides a more uniform tubular geometry, thereby offering a more suitable environment for further endothelial cell seeding.

3.2. 3D culture of HDFs in microvascular tissue constructs
To evaluate the ability of the microvascular networks to support cells in the GelMA hydrogel matrix, HDFs were mixed with the GelMA precursor solution prior to photo-crosslinking. After casting and cross-linking, the cell-containing microvascular constructs were perfused with the DMEM cell culture medium, cells were maintained through perfusion at <2 µl min⁻¹, with fresh medium every 12 h. Control structures, without vasculature were prepared using the identical GelMA cell suspension and cross-linking procedure, and cultured under identical conditions to the vascularised constructs.

To examine cell morphology and arrangement of HDFs in the vascularised tissue constructs, fluorescence imaging with phalloidin and DAPI was performed after 5 and 21 d in culture. Initially,
HDFs were encapsulated in GelMA hydrogel and displayed a rounded shape, scattered evenly within the hydrogel matrix. After 5 d in culture, HDFs appeared spindle-shaped with filopodia-like protrusions (figure 5(A)). These features became more obvious after 21 d in culture and as shown in figure 5(B), cells colonized the entire hydrogel matrix, forming a densely populated structure. Identical results were observed with the GelMA constructs containing the smaller 30 µm diameter vascular channels (figure 6(C)). HDFs extended along the surface of the microchannels. Preferential spreading around the
microchannels is likely related to the cells following contact guidance. The proximity of nutrient supply may also have promoted cells to migrate into the microchannel regions. Meanwhile, the actin microfilaments of HDFs were found to wrap around the channel walls forming 'bridges' across the microchannels. This phenomenon is likely because the microchannel walls act as physical obstacle for cells to grow in the perpendicular direction, which is consistent with other reports on topography-induced cell alignment [42].

Live/dead viability assays were performed over 21 d in 3D culture comparing the behaviour of the HDFs embedded in GelMA with 60 µm channels and those cultured in an unvascularised GelMA structure of identical external dimensions. The HDFs embedded in the vascular constructs exhibited high viability after 21 d in culture and formed dense cellular structures (figure 6(A)). There was a notable difference in the viability between the tissue constructs with and without microvasculature (figure 6(B)). Cells in the GelMA matrix without vascular channels died quickly and showed a clear reduction in viability with increasing time in culture, eventually only 13% of live cells remained after 21 d. Cells embedded in the microvascular tissue constructs showed a high viability (>90%) over 21 d culture. An analysis of variation (one-way ANOVA) was performed across the time points of the assay to test whether there was any variation in viability. The ANOVA returned \( p = 0.961 \), this is considerably greater than the threshold for significance \( (p < 0.05) \) hence there was no reason

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**Figure 6.** Cell viability assay of HDFs embedded in the engineered 60 µm channel diameter microvascular patterned tissue constructs cultured over 21 d. (A) Fluorescence microscopy images showing live (green) and dead (red) cells, confirming high level of cell viability. (B) Cell viability in vascular GelMA constructs compared with control unvascularised GelMA.

**Figure 7.** Fluorescent images of RFP-HUVECs seeded into 60 µm diameter microchannels showing uniform dispersion of HUVECs after seeding.
to reject the null hypothesis. Although no live/dead assay was carried out with the cells in the GelMA with 30 μm diameter vasculature, comparison of the cell density and morphology between cells grown in both vascularised sample (figures 5(B) and (C)) found no discernible difference. The fact that the presence of an artificial perfused vascular network permits the maintenance of cells in 3D culture supports the hypothesis that the presence of such a network is a necessary component of a fabricated prototissue.

3.3. Endothelialization of microvascular tissue constructs

An endothelialization process was performed by injecting HUVECs into the microchannels. This is illustrated in figure 7 through the use of RFP-HUVECs to visualize the injection process allowing imaging of the dynamic perfusion of cells. The seeded HUVECs were maintained at a perfused flow rate <2 μl min⁻¹, with a change in medium every 12 h. After 3 d in culture, HUVECs were stained by

Figure 8. Endothelialization of microvascular constructs. HUVECs were perfused into the microchannels with smallest feature size (A) 30 μm and (B) 60 μm and cultured for 3 d. Phalloidin was used to stain F-actin (red) and DAPI was used to stain the nuclei (blue).
phalloidin (Alexa Fluor 594) and DAPI for fluorescence imaging. As shown in figure 8, HUVECs formed a confluent endothelial layer that covered the entire surface of the microchannel network. HUVECs exhibited a cobble-stone pattern in keeping with a cellular syncytium. Typically, HUVECs were observed with a venule-like morphology at the smallest branching channels of 30 µm and 60 µm in each construct design, respectively, which was likely induced by the low flow shear stress [43].

In addition, to demonstrate the barrier function of the confluent endothelial layer, we perfused FITC-dextran solution through the endothelialized microchannels to aid the visualization of flow. The plan view of the constructs, as shown in figures 9(A) and (B), demonstrated the fluorescent dextran flow in the endothelialized microchannels, displayed an irregular line profile created by the presence of the ECs lining the channel. This is in contrast to the non-endothelialized bare GelMA constructs, where the fluorescent dextran flow shows a well-defined regular profile. It is believed that HUVECs formed an endothelial layer attached to the inner wall of the microchannels, which acts as a barrier between the flow and the hydrogel matrix. The presence of nuclei in the cells leads to the non-uniform thickness of the endothelium, thereby forming the irregular line profile. Meanwhile, from the cross-sectional view of the microchannels, lumens were clearly visualized by F-actin, nucleus and the FITC-dextran solution showing an arched lumen shape (figures 9(C) and (D)). Notably, unlike lithographic methods that create rectangular channels with right angles, these half cylindrical channels with rounded corners fabricated by multilayer EHD inkjet printing provides a more uniform tubular geometry, thereby offering a more suitable environment for the seeding of ECs [44].

3.4. 3D co-culture of GFP-HDFs and RFP-HUVECs in microvascular tissue constructs

To mimic the multiple cell types, present in native vascularised tissue, we carried out co-culture of GFP-HDFs and RFP-HUVECs in the engineered microvascular tissue constructs. GFP-HDFs were encapsulated in the GelMA hydrogel patterned with microvascular networks, followed by the endothelialization with RFP-HUVECs. The cell seeded structures were maintained by perfusing 1:1 ratio of endothelial growth medium (EGM-2 medium) and HDF growth medium (DMEM plus 10% (vol/vol) FBS) in the microvascular networks. After 7 d culture the GFP-HDFs spread into and colonized the
surrounding hydrogel matrix to form a dense proto-tissue (figure 10). The encapsulated GFP-HDFs produced reticular actin fibres wrapping around the microchannels acting as a support structure. Meanwhile, HUVECs formed a confluent endothelium layer along the direction of microchannels. On cross-section view, GFP-HDFs showed a typical spreading confluent spindle cell morphology, which is likely due to their preference for surface adherence. RFP-HUVECs formed a monolayer of ECs along the lumens of microchannels.

Finally, we developed a complete microvascular tissue mimic by perfusing fluorescent dextran solution into a GFP-HDFs/RFP-HUVECs co-culture construct. To avoid colour overlap with fluorescent cells, dextran was chosen with wavelength at 647 nm (far-red) and marked as blue pseudocolour in the image post adjustments. Fluorescent dextran solution represents blood flow perfused through the microvascular networks, in which GFP-HDFs and RFP-HUVECs formed supportive prototissue and endothelium, respectively (figure 11). This engineered 3D tissue construct simulates the basic components of a perfusable microvasculature, including supportive ECM, viable tissue cells, a functional barrier endothelial layer, and blood flow; each of them was represented by GelMA hydrogel, GFP-HDFs, RFP-HUVECs and fluorescent dextran solution respectively.

We consider whether EHD inkjet printing can further push the limits of feature resolution for biofabrication. It is clear from this work that we are capable of printing tissue mimicking hydrogel structures with a vasculature diameter of 30 µm that can be successfully endothelialized and subsequently perfused to maintain cells in 3D culture. In principle the feature resolution can be pushed the required factor of five smaller to achieve printed structures with dimensions comparable to physiological capillary beds, although it is not clear whether channels of this scale could be endothelialized successfully by cell suspension perfusion without blockage. A fundamental issue with reducing the scale of feature resolution is that the to maintain deposition rate, the drop generation frequency must scale with the inverse of drop volume and thus build times may become excessive. Another limitation to EHD deposition is the sensitivity of the process to the local electric field, and hence the substrate electrical properties and the nozzle stand-off distance. This may limit the accuracy of deposition when there is significant heterogeneity in material composition during additive layer printing or non-planarity in the substrate surface.

Finally, this is a preliminary platform that will be used to explore vascular development and biology. It is noted that vascular sprouting and neoangiogenesis beyond the construct channels has not
been seen at 21 d. Future investigations aim to prolong the duration of study and add additional biological triggers to try and develop the tissues further by means of additional growth factor signalling [45], mechanical forces [46] and other cells that facilitate vascular development [47].

4. Conclusions

To conclude, we have presented a manufacturing method based on high resolution EHD inkjet printing to fabricate hydrogel-based microvascular tissue constructs. The method integrates the printing/casting process, offering superior spatial resolution, flexibility and hydrogel compatibility over conventional bioprinting approaches. Microvascular structures can be fabricated with feature size down to 30 µm to mimic capillary vessels in the native arteriovenule connections. Cells seeded in the microvascular constructs exhibited their typical morphologies and high cell viability after up to 21 d culture for both fibroblasts and ECs when compared to constructs without microvascular channels. Heterogeneous tissue constructs with multiple cell types that are capable of long-term maintenance have been fabricated to replicate the basic composition and functions of the native microvasculature. Future utility of this approach can develop precise microvascular anatomicies, and incorporate a range of different cell types to create bespoke vascularized tissues for drug testing or even tissue replacement. The current platform opens up a number of new avenues for fabricating and investigating engineered functional tissues.

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References

[1] Atala A, Bauer S B, Soker S, Yoo J J and Retik A B 2006 Tissue-engineered autologous bladders for patients needing cystoplasty Lancet 367 1241–6

[2] Macchiarini P et al 2008 Clinical transplantation of a tissue-engineered airway Lancet 372 2023–30

[3] Kinstlinger I S and Miller J S 2016 3D-printed fluidic networks as vasculature for engineered tissue Lab. Chip. 16 2025–43

[4] Hasan A, Paul A, Vrana N E, Zhao X, Memic A, Hwang Y S, Dokmeci M R and Khademhosseini A 2014 Microfluidic techniques for development of 3D vascularized tissue Biomaterials 35 7308–25

[5] Sarker M D, Naghieh S, Sharma N K and Chen X 2018 3D biofabrication of vascular networks for tissue regeneration: a report on recent advances J. Pharm. Anal. 8 277–96

[6] Takêi T, Sakai T and Yoshida M 2016 In vitro formation of vascular-like networks using hydrogels J. Biosci. Bioeng. 122 519–27

[7] Nomi M, Atala A, De Coppi P and Soker S 2002 Principles of neovascularization for tissue engineering Mol. Aspects Med. 23 463–83

[8] Sarker M, Naghieh S, McLinnes A D, Schreyer D J and Chen X 2018 Strategic design and fabrication of nerve guidance conduits for peripheral nerve regeneration Biotechnol. J. 13 1700635

[9] Fernandez C E, Achneck H E, Reichert W M and Truskey G A 2014 Biological and engineering design considerations for vascular tissue engineered blood vessels (TEBVs) Curr. Opin. Chem. Eng. 3 83–90

[10] Frerich B, Lindemann N, Kurtz-Hoffmann J and Oertel K 2001 In vitro model of a vascular stroma for the engineering of vascularized tissues Int. J. Oral Maxillofac. Surg. 30 414–20

[11] Sarker M, Chen X B and Schreyer D J 2015 Experimental approaches to vascularisation within tissue engineering constructs J. Biomater. Sci. Polym. Ed. 26 683–734

[12] Udan R S, Culver J C and Dickinson M E 2013 Understanding vascular development Wiley Interdiscip. Rev. Dev. Biol. 2 327–46

[13] Mironov V, Reis N and Derby B 2006 Bioprinting: a beginning Tissue Eng. 12 633–34

[14] Derby B 2012 Printing and prototyping of tissues and scaffolds Science 338 921–2

[15] Murphy S V and Atala A 2014 3D bioprinting of tissues and organs Nat. Biotechnol. 32 773–85

[16] Kolesky D B, Truby R L, Gladman A S, Busbee T A, Homan K A and Lewis J A 2014 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs Adv. Mater. 26 3126–30

[17] Bertassoni I E et al 2014 Hydrogel bioprinted microchannel networks for vascularization of tissue engineering constructs Lab. Chip. 14 2202–11

[18] Kolesky D B, Homan K A, Skylar-Scott M A and Lewis J A 2016 Three-dimensional bioprinting of thick vascularized tissues Proc. Natl. Acad. Sci. 113 3179–84

[19] Miller J S et al 2012 Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues Nat. Mater. 11 768–74

[20] Chan V, Zorlutuna P, Jeong I H, Kong H and Bashir R 2010 Three-dimensional photopatterning of hydrogels using stereolithography for long-term cell encapsulation Lab. Chip. 10 2062–70

[21] Arcateau K, Mann B K and Wicker R B 2006 Stereolithography of three-dimensional bioactive poly(ethylene glycol) constructs with encapsulated cells Ann. Biomed. Eng. 34 129–41

[22] Zhang A P, Qu X, Soman P, Hribar K C, Lee J W, Chen S and He S 2012 Rapid fabrication of complex 3D extracellular microenvironments by dynamic optical projection stereolithography Adv. Mater. 24 4266–70

[23] Tumbleston J R et al 2015 Continuous liquid interface production of 3D objects Science 347 1349–52

[24] Zhu W et al 2017 Direct 3D bioprinting of prevascularized tissue constructs with complex microarchitecture Biomaterials 124 106–15

[25] Grigoryan B et al 2019 Multivascular networks and functional intravascular topologies within biocompatible hydrogels Science 364 458–64

[26] Omes M S, Sutanto E, Ferreira P M, Alleyne A G and Rogers J A 2015 Mechanisms, capabilities, and applications of high-resolution electrohydrodynamic jet printing Small 11 4237–66

[27] Choi H K, Park J-U, Park O O, Ferreira P M, Georgiadis J G and Rogers J A 2008 Scaling laws for jet pulsations associated with high-resolution electrohydrodynamic printing Appl. Phys. Lett. 92 123109

[28] Derby B 2010 Inkjet printing of functional and structural materials: fluid property requirements, feature stability, and resolution Ann. Rev. Mater. Res. 40 395–414

[29] Yarin A L, Koombhongse S and Reneker D H 2001 Taylor cone and jetting from liquid droplets in electrospinning of nanofibers J. Appl. Phys. 90 4836–66

[30] Stringer J and Derby B 2010 Formation and stability of lines produced by inkjet printing Langmuir 26 10365–72

[31] Park J U, Lee J H, Paik U, Lu Y and Rogers J A 2008 Nanoscale patterns of oligoamines formed by electrohydrodynamic jet printing with applications in biosensing and nanomaterials assembly Nano Lett. 8 4210–6

[32] Kim S Y et al 2016 High-resolution electrohydrodynamic inkjet printing of stretchable metal oxide semiconductor transistors with high performance Nanoscale 8 17113–21

[33] Kim B H et al 2015 High-resolution patterns of quantum dots formed by electrohydrodynamic jet printing for light-emitting diodes Nano Lett. 15 969–73

[34] Galliker P, Schneider J, Eghlidi H, Kress S, Sandoghdar V and Poulikakos D 2012 Direct printing of nanomaterials by electrostatic autofocussing of ink nanodroplets Nat. Commun. 3 890

[35] Schneider J, Rohner P, Galliker P, Raja S N, Ying P, Tiwari B M and Poulikakos D 2015 Site-specific deposition of single gold nanoparticles by individual growth in electrophoretically-printed attoliter droplet reactors Nanoscale 7 9510

[36] Nie J, Gao Q, Xie C, Lv S, Qiu J, Liu Y, Guo M, Guo R, Fu J and He Y 2019 Construction of multi-scale vascular chips and modelling of the interaction between tumours and blood vessels Mater. Horiz. 7 82–92

[37] Fairbanks B D, Schwartz M P, Bowman C N and Anseth K S 2009 Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphinate: polymerization rate and cytocompatibility Biomaterials 30 6767–73

[38] Majima T, Schnabel W and Weber W 1991 Phenyl-2,4,6-trimethylbenzoylphosphinates as water-soluble photoinitiators. Generation and reactivity of O (⋅OH) (O−) radical anions Die Makromol. Chem. 192 2307–15

[39] Poncio E, Barthes J, Bour J, Michel M, Bertani P, Hemmerlé J, D’Ischia M and Ball V 2016 Oxidant control of polydopamine surface chemistry in acids: a mechanism-based entry to superhydrophilic–superoleophobic coatings Chem. Mater. 28 4697–705

[40] Lee J N, Jiang X, Ryan D and Whitesides G M 2004 Compatibility of mammalian cells on surfaces of poly(dimethylsiloxane) Langmuir 20 11684–91

[41] Johnson L M, Gao L, Shields IV C, Smith M, Efimenko K, Cushing K, Genzer J and López G P 2013 Elasticomic microparticles for acoustic mediated biooperations J. Nanobiotechnol. 11 2

[42] Li H, Weng Y S, Wen F, Ng K W G, Ng K L K, Venkatraman S S, Boey F Y C and Tan L P 2013 Human mesenchymal stem-cell behaviour on direct laser micropatterned electrop spun scaffolds with hierarchical structures Macromol. Biosci. 13 299–310

[43] Chien S 2008 Effects of disturbed flow on endothelial cells Ann. Biomed. Eng. 36 554–62
[44] Green J V, Kniazeva T, Abedi M, Sokhey D S, Taslim M E and Murthy S K 2009 Effect of channel geometry on cell adhesion in microfluidic devices Lab. Chip. 9 677–85
[45] Moccia F, Negri S, Shekha M, Faris P and Guerra G 2019 Endothelial Ca\textsuperscript{2+} signaling, angiogenesis and vasculogenesis: just what it takes to make a blood vessel Int. J. Mol. Sci. 20 5962
[46] Gordon E, Schimmel L and Frye M 2020 The importance of mechanical forces for \textit{in vitro} endothelial cell biology Front. Physiol. 11 684
[47] Britto D D, Wyroba B, Chen W, Lockwood R A, Tran K B, Shepherd P R, Hall C J, Crosier K E, Crosier P S and Astin J W 2018 Macrophages enhance Vegfa-driven angiogenesis in an embryonic zebrafish tumour xenograft model Dis. Model. Mech. 11 dmm035998
[48] Lee B H, Shirahama H, Cho N J and Tan L P 2015 Efficient and controllable synthesis of highly substituted gelatin methacrylamide for mechanically stiff hydrogels RSC Adv. 5 106094–7