Biofabrication

PAPER

In vitro vascularization of hydrogel-based tissue constructs via a combined approach of cell sheet engineering and dynamic perfusion cell culture

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OPEN ACCESS

RECEIVED
12 June 2022

REVISED
29 August 2022

ACCEPTED FOR PUBLICATION
22 September 2022

PUBLISHED
27 October 2022

Keywords: angiogenesis, cell sheet engineering, perfusion cell culture, thermoresponsive surface, vascularization, vat photopolymerization

Supplementary material for this article is available online

Abstract

The bioengineering of artificial tissue constructs requires special attention to their fast vascularization to provide cells with sufficient nutrients and oxygen. We addressed the challenge of *in vitro* vascularization by employing a combined approach of cell sheet engineering, 3D printing, and cellular self-organization in dynamic maturation culture. A confluent cell sheet of human umbilical vein endothelial cells (HUVECs) was detached from a thermoresponsive cell culture substrate and transferred onto a 3D-printed, perfusable tubular scaffold using a custom-made cell sheet rolling device. Under indirect co-culture conditions with human dermal fibroblasts (HDFs), the cell sheet-covered vessel mimic embedded in a collagen gel together with additional singularized HUVECs started sprouting into the surrounding gel, while the suspended cells around the tube self-organized and formed a dense lumen-containing 3D vascular network throughout the gel. The HDFs cultured below the HUVEC-containing cell culture insert provided angiogenic support to the HUVECs via molecular crosstalk without competing for space with the HUVECs or inducing rapid collagen matrix remodeling. The resulting vascular network remained viable under these conditions throughout the 3 week cell culture period. This static indirect co-culture setup was further transferred to dynamic flow conditions, where the medium perfusion was enabled via two independently addressable perfusion circuits equipped with two different cell culture chambers, one hosting the HDFs and the other hosting the HUVEC-laden collagen gel. Using this system, we successfully connected the collagen-embedded HUVEC culture to a dynamic medium flow, and within 1 week of the dynamic cell culture, we detected angiogenic sprouting and dense microvascular network formation via HUVEC self-organization in the hydrogel. Our approach of combining a 3D-printed and cell sheet-covered vascular precursor that retained its sprouting capacity together with the self-assembling HUVECs in a dynamic perfusion culture resulted in a vascular-like 3D network, which is a critical step toward the long-term vascularization of bioengineered *in vitro* tissue constructs.

1. Introduction

Bioengineered *in vitro* tissue constructs are widely used both in regenerative medicine to aid tissue repair *in vivo* and in the development of tissue models to replace or add to existing *in vivo* animal models. However, growing a tissue mimic is still a great challenge, as it requires sufficient vascularization to ensure efficient oxygen and nutrient supply to cells. Several approaches to vascularizing 3D tissue constructs have been introduced via various *in vitro* tissue engineering techniques [1, 2],

including biofabrication of patterned channels of vascular endothelial cells inside a hydrogel structure [3–5] or via channel templating and reseeding with vascular endothelial cells throughout a tissue scaffold [6]. Even though these endothelialized channels can mature into biomimetic vascular vessels within a native tissue, they can only reach a limited number of cells in the *in vitro* tissue construct, thereby requiring further microvascularization. This can be accomplished by implanting the *in vitro* engineered tissue construct into a living host system for a dedicated amount of time, after which neovascularization within the transplanted tissue is ideally observed as well as its anastomosis to the host circulation [7]. However, even under *in vivo* conditions, vascular growth from the host tissue triggered by the natural inflammatory and regenerative wound healing response is only several tens of micrometers per day, which alone is not sufficient for the *in vivo* vascularization of thicker 3D tissue constructs [8]. Therefore, both *in vivo* and *in vitro* vascularization of an entire bioengineered 3D tissue construct remains challenging.

An attractive approach toward the *in vitro* vascularization of 3D tissue constructs is to take advantage of cell sheet engineering, a technique originally introduced by Okano and co-workers [9, 10]. In typical cell sheet engineering techniques, cells are cultured until the full confluency on a thermoresponsive surface that can undergo a transition from a hydrophobic, cell-adhesive state to a hydrophilic, cell-repellent state upon temperature decrease from 37 °C to room-temperature (RT) [11]. When this confluently grown cell monolayer on a functional thermoresponsive substrate is transferred outside a cell culture incubator, the whole cell sheet is mildly detached and can be harvested as a single layer together with its intact extracellular matrix [12–15]. Previously, a few groups have used cell sheets for building vascularized tissue constructs [16–19] as well as for the fabrication of large to medium-diameter tissue-engineered vascular grafts [20, 21]. Asakawa *et al* [16] induced vascularization of fibroblast sheets by sandwiching them together with a human umbilical vein endothelial cell (HUVEC) sheet, and Sekine *et al* [17] stacked cell sheets derived from a co-culture of rat cardiac and endothelial cells. In addition to endothelial cell sheets, thin, microstructured vascular smooth muscle sheets and patches have been produced for vascular tissue engineering applications [18, 19]. Although piling up cell sheets with the intended self-organization of endothelial cells into vascular structures is a feasible method for the fabrication of very thin tissue layers, it would not be a feasible method for thicker tissue mimics [17].

In contrast to patches made of thin cell sheet stacks, the use of self-standing cell sheet-covered vascular precursors with defined access ports could enable *in vitro* vascularization of thicker tissue constructs under subsequent exposure to proangiogenic conditions. As an example of such vascular mimics of a medium-sized diameter, Ahn *et al* [20] fabricated non-endothelialized vascular tubes by manually wrapping a sheet of sheep vascular smooth muscle cells around an electrospun poly(ε-caprolactone) (PCL)/collagen tube (inner diameter: 4.75 mm). Similarly, Hibino *et al* [21] folded a differentiated induced pluripotent stem cell sheet around a poly(glycolic acid) tube (inner diameter: 0.8 mm) to obtain tissue-engineered vascular grafts. Furthermore, Kang *et al* [22] wrapped a prevascularized co-culture sheet of human mesenchymal stem cells and HUVECs around a porous tricalcium phosphate scaffold (outer diameter: 4 mm), although this was not used as a vascular precursor but rather as a vascularized bone-tissue construct. However, in these studies, no long-term vascularization of 3D tissue constructs via engineered perfusable vascular mimics was explored.

In the current study, we aimed to replicate a native vascular bed by embedding a HUVEC sheet-covered perfusable vascular mimic into a collagen matrix together with suspended, self-assembling HUVECs. The small-diameter (outer/inner diameter: 1.5/0.5 mm) tubular scaffold was 3D printed with vat photopolymerization using a hybrid resin of gelatin methacryloyl (GelMA) and PCL-MA, and a confluent cell sheet of HUVECs was subsequently rolled around the tube using a customized rolling device. The formation of a vascular-like network of HUVECs embedded in collagen hydrogel was followed for 3 weeks in an indirect co-culture with human dermal fibroblasts (HDFs). Furthermore, a new perfusion cell culture circuit was established to validate the vascular network formation under physiologically more relevant dynamic cell culture conditions.

2. Material and methods

2.1. Materials

Gelatin from porcine skin (gel strength 300, type A, 50–100 kDa, Sigma-Aldrich), ε-caprolactone (Aldrich), tin(II) 2-ethylhexanoate ($Sn(Oct)_2$, Aldrich, 92.5%), di(trimethylolpropane) (diTMP, Aldrich, 97%), methacrylic anhydride (Aldrich, 94%), formamide (99.5%, Roth), ethyl(2,4,6-trimethylbenzoyl) phenylphosphinate (also known as Lucirin TPO-L) photoinitiator (Fluorochem), and Orasol Yellow 2RLN dye (Kremer Pigmente) were used as received. Green fluorescent protein (GFP)-tagged HUVECs were purchased from Cellworks (Caltag Medsystems Company) and non-tagged HUVECs were purchased from Cellsystems®. HDFs were isolated from juvenile human foreskin (age between 5 and 18 months) under the ethical approval of the Ethics Committee at Charité Universitätsmedizin Berlin, Germany

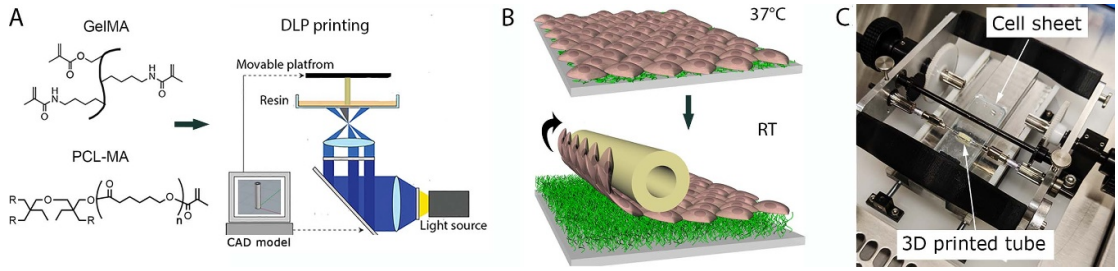

Figure 1. Schematic presentation of (A) chemical structures of GelMA and PCL-MA in the hybrid resin and DLP 3D printing and (B) HUVEC sheet detachment from a thermoresponsive tissue culture slide and subsequent cell sheet rolling. (C) Photograph of the custom-made cell sheet rolling device.

(EA1/081/13) using a collagenase/dispase-based protocol, as previously described [23]. High glucose Dulbecco's modified Eagle Medium (DMEM, Gibco®), fetal bovine serum (FBS, Biochrom), penicillin-streptomycin (Life Technologies), and supplemented Vasculife® vascular endothelial growth factor (VEGF) medium (LifeLine Cell Technology) were used in cell culture studies as received unless otherwise noted. SpectraPor® dialysis tubing made from regenerated cellulose with a molecular weight cutoff (MWCO) of 3.5 kDa was purchased from Carl Roth GmbH + Co. KG (Karlsruhe). Colorimetric MTS solution (Abcam), Hoechst 33342 DNA-intercalating dye (Thermo Fisher), a LIVE/DEAD® cell viability kit (Invitrogen®), and green 5-chloromethylfluorescein diacetate dye (CMFDA, Thermo Fisher) were used in cell culture, as described in the Methods section.

2.2. Synthesis of photocrosslinkable macromers, formulation of the resin, and 3D printing of a perfusable tube by vat photopolymerization

For the 3D printing of flexible and durable small-diameter tubes, a photocrosslinkable 3D printing resin of GelMA and four-arm PCL-MA for vat photopolymerization (figure 1(A)) was formulated as previously described [24]. Briefly, GelMA and PCL-MA in the ratio of 70/30 wt-% were dissolved in formamide (50 wt-%) and mixed with visible light sensitive 2 wt-% of ethyl(2,4,6-trimethylbenzoyl) phenylphosphinate photoinitiator and 0.05 wt-% of Orasol Yellow dye to enable the highest spatial control over photocrosslinking. To 3D print a porous tube for rolling cell sheets, a CAD model of a hollow tube with an outside diameter of 1.5 mm, an inside diameter of 0.5 mm, and a length of 1 cm was designed using Rhinoceros 5 software. A digital light processing (DLP) 3D printer (Titan 2 from Kudo3D, Taiwan) was equipped with a UV-filtered projector (Acer Full HD 1080) that projected 1920 × 1080 pixels of visible light onto the bottom of a resin reservoir to crosslink the desired layer thickness. The crosslinking time was 25 s for a 100 μm layer, and the temperature inside the printing hood was maintained at 32 °C with an additional set of a heater and a thermostat (IncuKit^{TM} Mini, Incubator Warehouse). After 3D printing, the residual photoinitiator, non-crosslinked macromer, orange dye, and solvent were removed from the samples by extraction with ethanol for 3 days and water for 7 days.

2.3. Cell culture

Both GFP-labeled and unlabeled HUVECs (p 3–8) were cultured in Vasculife® VEGF endothelial medium with Vasculife® supplements (2% FBS), and HDFs (p 3–8) were cultured in high-glucose DMEM mixed with 10% FBS and 1% penicillin/streptomycin. Before seeding, all the cells were cultured under standard conditions (5% CO_2, 37 °C, 95% humidity) until the maximum of 70%–80% confluency. To study the stability of 3D-printed GelMA/PCL-MA hydrogels in a cell culture, HUVECs (5×10^4 cells cm^{-2}) were seeded on top of the photocrosslinked hydrogels (diameter of 5 mm, thickness of 0.3 mm) that had been immersed in 70% ethanol for 30 min and twice in PBS for 15 min. At determined time points of the cell culture, triplicate samples were incubated in trypsin for 10 min, and after washing with PBS, the samples were lyophilized and weighted to give their remaining mass.

2.4. Cell sheet formation and the rolling process

For cell sheet formation, thermoresponsive poly(glycidyl methyl ether-*co*-ethyl glycidyl ether) (PGE, M_n = 30 kDa, monomer ratio of 1:3) functionalized with a short benzophenone anchor block was synthesized and self-assembled from a selective solvent as a monolayer on a polystyrene (PS) slide (7.5 cm × 2.5 cm × 0.15 cm), as previously described [13, 23]. For the coating with PGE, the transparent PS slide was immersed in an aqueous/ethanolic polymer solution for 1 h (0.25 mg ml^{-1} of PGE in 45% EtOH in H_2O) and then irradiated with UV-light (UV-KUB 2, KLOE) for 160 s. The coated slides were disinfected with 70% ethanol for 10 min and rinsed twice with cold PBS, after which a silicone frame (2 cm × 3 cm) was placed on the slide and a HUVEC suspension (5.1×10^5 cells in 2 ml of medium) was pipetted inside the frame. The cells were cultured in media supplemented with 2% FBS for the first day and with 10% FBS for a further 3 days. After four

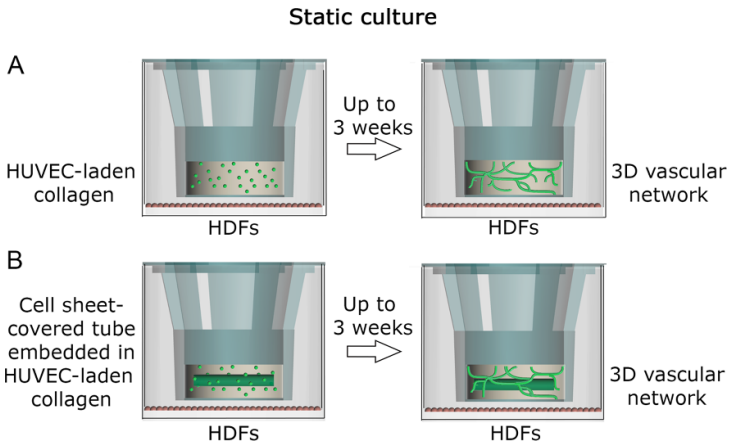

Figure 2. Schematic presentation of the setup to vascularize collagen hydrogels under static conditions in a membrane insert culture (A) without and (B) with a cell sheet-covered tube.

days of cell culture, the cell-covered slide was transferred from warm medium to RT PBS for 10 min to partly detach the confluent cell layer from its edge (figure 1(B)). The sheet was then rolled around a 3D-printed tube during detachment using a custom-made rolling device developed and prototyped by Ospin GmbH (Berlin, Germany) (figure 1(C)). The GelMA/PCL-MA tube was connected to the rolling device via a thin (diameter of 0.55 mm) stainless-steel rod placed inside the tube, which enabled an easy transfer of the cell-covered tube from the rolling device to the collagen matrix. To prevent contamination, the rolling device was wiped before its use with 70% ethanol, and all parts in contact with the GelMA/PCL-MA tube were disinfected for 15 min in 70% ethanol and rinsed twice with sterile PBS.

2.5. Network formation in a static culture of a 3D collagen construct

To form a cell-laden collagen solution (2.5 mg ml^{-1}), acidic rat tail collagen solution (500 μl, 5 mg ml^{-1} in 20 mM acetic acid) was neutralized with 10× PBS (100 μl) and 0.1 M NaOH (110 μl) and mixed with a HUVEC suspension in Vasculife® medium supplemented with 0.5% FBS (290 μl, 2 × 10^6 cells ml^{-1}). Vascular network formation was studied under both static and dynamic conditions. GFP-HUVECs were used in the static insert culture to enable easy detection of the cells in the collagen gel, whereas in the dynamic culture, network formation was validated with unlabeled HUVECs. For the static culture, an acellular collagen layer was first prepared by solidifying collagen solution (160 μl) in a 12-well plate Transwell® insert (Corning® Costar®, 0.4 μm pore size) for 30 min at 37 °C. After that, additional collagen/cell suspension (200 μl) was placed on the hydrogel surface either without (figure 2(A)) or with (figure 2(B)) a rolled HUVEC cell sheet around a 3D-printed tube. The second collagen layer was solidified for 40 min at 37 °C. After gelation, Vasculife® medium (500 μl) containing 0.5% FBS was added on top of the gel, and the insert was transferred to a 12-well suspension culture plate (Greiner Bio-One CELLSTAR®), where HDFs (25 000 cells cm^{-2}) were seeded in DMEM (1.5 ml) a day before. The medium inside and outside the insert was changed every 3 days.

2.6. Chamber design and dynamic culture for the network formation

For the dynamic vascularization culture, two different perfusion chambers were designed: one to host the HUVEC sheet-covered tube together with suspended HUVECs in collagen (referred to here as a tube chamber), and the second to host the HDFs (i.e. a slide chamber). The chambers were included in a row in a dynamic culture system comprising two medium reservoirs and two peristaltic pumps to generate two constant fluid flows that were controlled using an open-source hardware platform comprising a microcontroller (Arduino Uno Rev 3) (figure 3). A detailed description of the 3D-printed tube chamber can be found in the supplementary information (figure S1). The tube chamber featured a confined collagen compartment (1 cm × 1 cm × 0.5 cm) with two sets of medium inlet and outlet connectors to enable medium flow both through the vascular tube and on top of the collagen hydrogel. The slide chamber hosting the HDFs was fabricated via computer numerical control (CNC) milling of bulk polycarbonate (Makroclear®, Arla Plast). It featured a flat compartment for an HDF monolayer (7.7 cm × 2.6 cm × 0.15 cm) with one set of medium inlet and outlet connectors to allow medium flow on top of the cells. The autoclavable and translucent tube chamber for hosting the collagen construct was CAD-modeled with Rhinoceros® software and 3D printed with an Orange

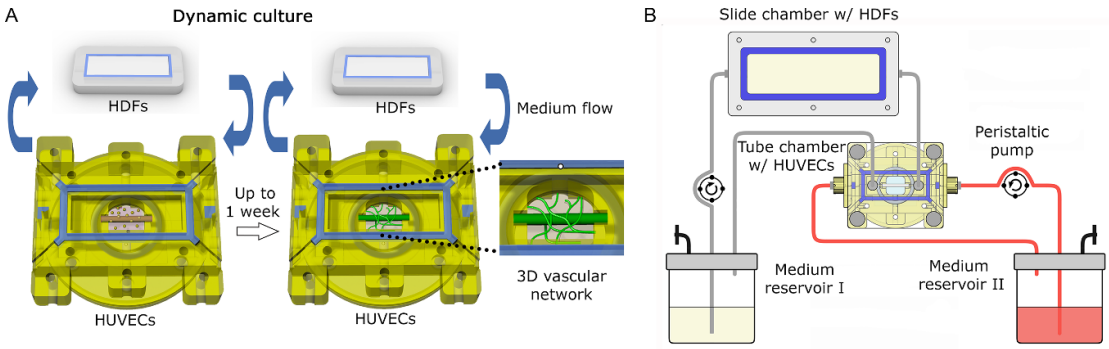

Figure 3. Schematic presentation of (A) the setup to vascularize collagen hydrogels under dynamic conditions in a 3D-printed chamber that allows dynamic medium perfusion through the construct and (B) the entire circuits for the dynamic cell culture: while the grey circuit delivers HDF-conditioned medium to the suspended HUVECs in the gel to induce and assist their self-assembly into a microvascular network, the red circuit allows independent perfusion of the cell sheet-covered tubular scaffold.

30 SLA printer (Longer3D) [25], using a biocompatible BioMed Amber Resin (Formlabs) with the burn-in time, range, and exposure time of 45 s, 0.25 mm, and 7.25 s, respectively. To enable real-time microscopy imaging of the HUVEC network formation in the collagen, the top lid of the tube chamber was made of transparent polycarbonate (Makroclear®, Arla Plast), and the center of the chamber bottom consisted of a circular glass cover slip. Furthermore, the top lid of the chamber contained screw threads to connect the inlet and outlet silicone hose for medium flow on top of the hydrogel. To construct the hydrogel, the bottom of the collagen compartment was first filled with a neutralized acellular collagen solution (500 μl, 2.5 mg ml^{-1}), which was allowed to solidify at 37 °C for 30 min, after which the tube with a rolled HUVEC sheet was placed between two needles on top of the hydrogel together with HUVEC-laden collagen solution (200 μl, 2.5 mg ml^{-1}, 2 × 10^6 cells ml^{-1}). The entire process of cell sheet rolling and the subsequent adjustment of the tube to the dynamic perfusion chamber can be seen in supplementary video S1. As a control sample for the dynamically cultured gels, HUVEC-laden Transwell® inserts with HUVEC medium both inside and outside the insert were prepared, as described above. After gelation of the collagen for 40 min at 37 °C, the tube in the dynamic samples was connected to perfusion of 8 ml of DMEM medium (0.7 ml min^{-1}) via syringe needles and silicone tubing (Tygon® 3350 and PharmaMed® BPT, Saint-Gobain Performance Plastic Corp.) using a peristaltic pump. Furthermore, the second set of inlet and outlet on top of the hydrogel were connected to perfusion with 8 ml of Vasculife® medium containing 0.5% of FBS (0.7 ml min^{-1}) that was first pumped through the slide chamber containing a TCPS slide (7.5 cm × 2.5 cm × 0.15 cm) on which HDFs (25 000 cells cm^{-2}) had been seeded a day before. After setting up the dynamic culture circuit, the entire system was transferred to a cell culture incubator maintained at the standard cell culture conditions (37 °C, 5% CO_2). CO_2-permeable silicone tubing was used in the circuit, and a hydrophobic filter was added on top of both medium reservoirs to allow gas exchange between the medium and the ambient air in the incubator. Therefore, the CO_2 content was expected to remain stable inside the cell culture chambers. The medium in both reservoirs was changed after the first 3 days of cell culture. To ensure the contamination-free culture, the assembled circuits for the dynamic culture, excluding the peristaltic pumps, were autoclaved before use.

2.7. Computational fluid dynamics (CFD) simulation

The numerical analysis of the flow conditions in the designed tube chamber was done with COMSOL Multiphysics software (v6.0, including a CFD module). First, the flow channels were modeled using the Rhinoceros 5 software and imported into COMSOL. Meshing was conducted using a COMSOL's physics-controlled sequence, where the gravity was included. The dynamic viscosity of the medium was chosen based on published data [26] with 0.73 mPa s for HUVEC medium with 0.5% FBS and 0.93 mPa s for DMEM with 10% FBS. Inlet flow rates were defined according to the experimental conditions with 0.7 ml min^{-1} for both circuits.

2.8. Stiffness of the collagen hydrogel

The storage modulus of the acellular collagen hydrogels after 24 h incubation in PBS at 37 °C was studied using an oscillating rheometer (Kinexus pro+, Malvern Panalytical) with an 8 mm parallel plate geometry. First, the viscoelastic regime of wet samples (d = 8 mm, h = 4 mm) was determined with an amplitude sweep from a strain of 0.1%–2% at 0.5 Hz, after which the storage modulus was measured with a frequency sweep from 0.1 to 3 Hz at 37 °C using a 0.5% strain. The final stiffness value was the average of three samples reported at a frequency of 0.5 Hz and 0.5% strain.

2.9. Preparation of samples for microscopy

The porous wall structure of the acellular 3D-printed tubes was visualized by scanning electron microscopy (SEM, Hitachi SU8030, 15 kV) after cutting 1 mm thick cross-sections of a lyophilized tube and making them electrically conductive by coating them with a thin (∼8–10 nm) gold layer in a sputter coater (Emscope SC 500, Quorum Technologies) under a high vacuum. To evaluate whether the HUVEC sheet covered the whole surface of the 3D-printed tube after rolling the cell sheet, the tube was incubated in MTS solution (10% in PBS) for 10 min at 37 °C to stain metabolizing, living cells with blue color, and after taking photographs, it was embedded in Tissue-Tek® cryomedia and shock-frozen by partly immersing the sample holder in liquid nitrogen. Thin layers (8 μm) of the frozen samples were sectioned using a cryotome at −16 °C (CM1510 S, Leica Biosystems). The cryosections were then fixed with 4% PFA for 20 min at RT, stained with Hoechst 33 342 (10 ng ml^{-1}) for 5 min at RT, and further visualized using a fluorescence microscope (Axio Observer Z1, Carl Zeiss). Self-organization and network formation of GFP-HUVECs in the collagen hydrogels were visualized during the course of the culture via fluorescence microscopy without additional staining, and at the endpoint of the cell culture via confocal laser scanning microscopy (LSM800, Carl Zeiss) or two-photon microscopy (TriMScope II, LaVision BioTec) after fixation with 4% PFA for 20 min at RT and staining with Hoechst 33342 (10 ng ml^{-1}) for 5 min at RT. The excitation source of the two-photon microscope was a near-infrared laser (Ti:Sa, Chameleon Ultra II, Coherent) tuned at 850 nm and 920 nm, repetition rate 80 MHz, and pulse width 140 fs. A water-immersion objective lens (20×, NA 1.05, Apochromat, Olympus) was used to focus the laser beam into the sample. The fluorescence signal was collected in the backward direction using a dichroic mirror (775, Chroma) and then passed through interference filters (466/60 nm and 525/50 nm) and finally detected by photomultiplier tubes (Hamamatsu). The untagged HUVECs used in the dynamic culture were fluorescence-stained with green CMFDA (10 μM in PBS) for 15 min at 37 °C before fixation with 4% PFA and image acquisition via confocal microscopy. For live/dead staining, HDFs were incubated with fluorescein diacetate (FDA, 10 μM) and/or propidium iodide (PI, 50 μM) for 5 min before image acquisition via fluorescence microscopy. For quantification of the viability of HDFs in the dynamic culture, cells were cultured as described above, and on day 7, the cells were trypsinized, centrifuged, and resuspended in PBS. Dead cells were stained by incubating the cells in 7.5 μM PI solution for 5 min, after which they were centrifuged, resuspended in PBS, and analyzed using a fluorescence-acivated cell sorting (FACS) flow cytometer (Attune NxT, Invitrogen). 20 000 events were measured with a 488 nm laser and a 585/40 emission filter.

2.10. Quantification of VEGF content in the HUVEC medium

Cell culture media from both static and dynamic cultures were collected after a 7 day cell culture and stored at −20 °C for further analysis. The VEGF Human ELISA Kit (Invitrogen) was used according to the manufacturer's protocol. Before the assay, the medium samples were diluted to half to maintain the VEGF concentration within the detection limit of the assay (15.6–1500 pg ml^{-1}). The absorbance of the samples was determined at 450 nm, and the values were translated to concentrations using the provided VEGF standard samples.

2.11. Statistical analysis

The statistical significance of the data was determined using the Kruskal–Wallis test followed by Dunn's multiple comparisons test ($p < 0.05$), provided by Prism 9 software (GraphPad, USA).

3. Results

3.1. 3D printing of porous tubes for cell sheet rolling

The small-diameter tubes for rolling cell sheets were 3D printed using GelMA/PCL-MA hybrid resin that was previously optimized for the visible light-based DLP printer. The concentration of 50% of GelMA/PCL-MA in formamide yielded a resin with suitable viscosity and crosslinking properties for the DLP when the printing temperature was maintained at 32 °C. The hollow 3D-printed tube with an outer diameter of 1.5 mm and an inner diameter of 0.5 mm (figure 4(A)) was flexible, durable, and easy to handle. SEM imaging of a cross-section of the lyophilized tube revealed a highly microporous structure of the 3D-printed material (figure 4(B)), which is needed to secure the medium and nutrient permeability of the tube wall. The mass of 3D-printed GelMA/PCL-MA hydrogels only slightly decreased when HUVECs were cultured on the samples for 3 weeks (figure 4(C)).

3.2. Cell sheet detachment and rolling around the 3D-printed tube

The 3D-printed tubular scaffold was covered with a HUVEC cell sheet to form a vessel mimic with high cell seeding density. For the cell sheet, HUVECs were cultured on a thermoresponsive PGE-coated PS slide for 4 days, until a dense, confluent monolayer was obtained. When the cell-covered slide was transferred from the incubator to PBS at RT, the sheet began to detach from its edges after 10 min (figure 5(A)). An early attempt to detach the HUVECs on day 3 instead of a full 4 day culture resulted in holes in the cell sheet despite their full confluency (figure S2),

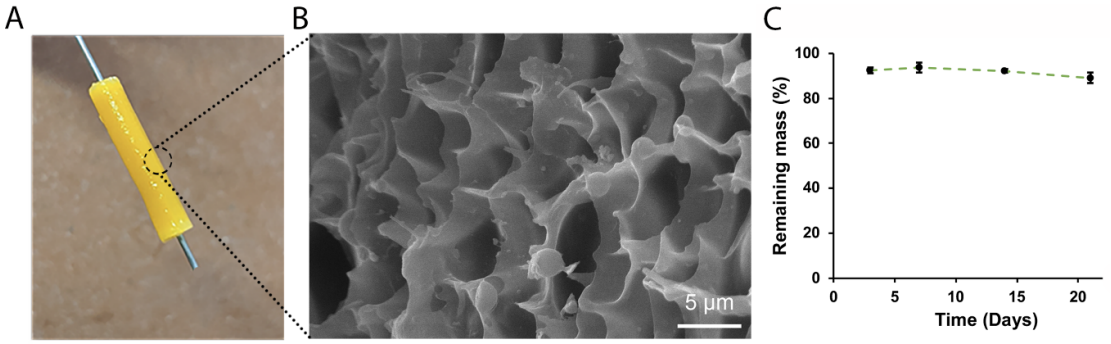

Figure 4. (A) Photograph of a 3D-printed GelMA/PCL-MA tube and (B) an SEM image of a cross-section of the lyophilized tube showing the porosity of the tube wall. (C) Remaining dry mass of 3D-printed GelMA/PCL-MA hydrogel within a 3 week cell culture study.

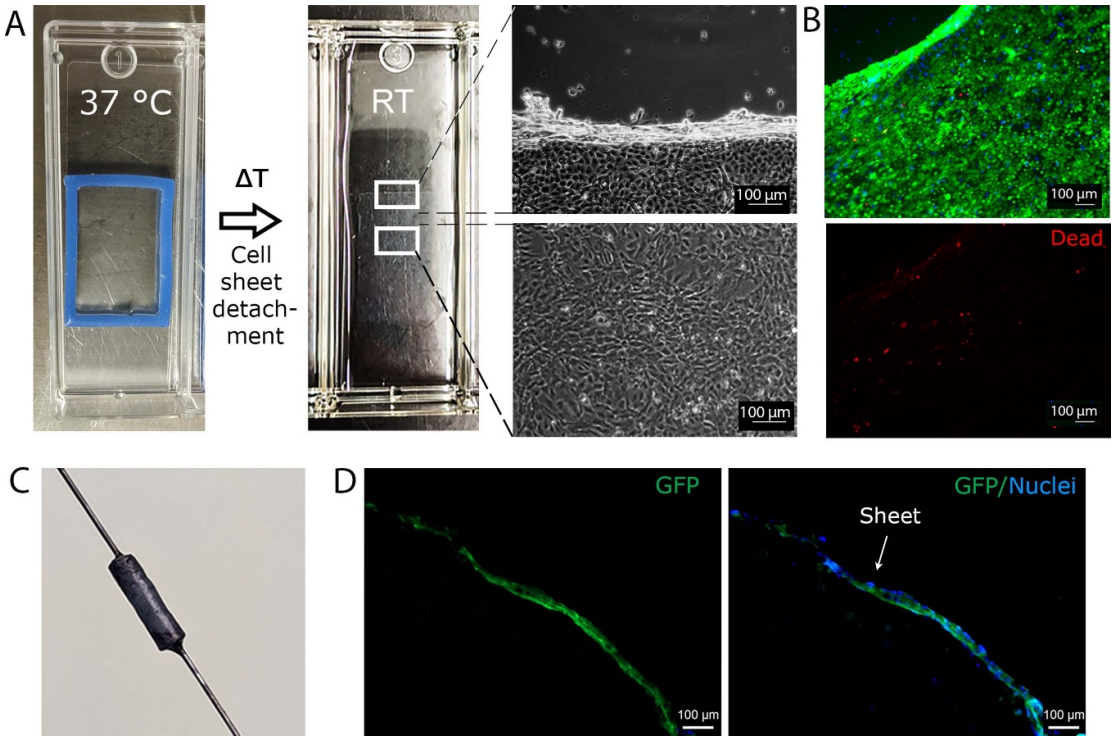

Figure 5. (A) Representative photographs of the cell sheet before and after the cell sheet detachment and microscopy images of the partial detachment of the sheet at its upper edge. (B) Fluorescent microscopy images of a PI-stained GFP-HUVEC (green) sheet showing only very few dead cells (red). (C) Photograph of an MTS-stained (blue) cell sheet rolled around a 3D-printed tube. (D) Representative fluorescence microscopy image of a cryo-cut of the cell-covered tube showing a GFP-HUVEC monolayer (green) and cell nuclei (blue, Hoechst staining) on the tube surface.

thereby emphasizing the importance of strong intercellular connections. The integrity of cell-cell contacts in the HUVEC monolayer on day 4 was verified by fluorescent staining of VE-cadherin and ZO-1 tight junction proteins between the cells (figure S3). Fluorescent staining of the GFP-HUVEC sheet with PI revealed only a few dead cells after full detachment (figure 5(B)). We used a custom-made rolling device to efficiently transfer the cell sheet during the detachment process onto the 3D-printed tube with the luminal side of the sheet facing the scaffold. Turning the 3D-printed GelMA/PCL-MA tube several times on top of the detaching cell sheet relocated the cells as a homogeneous layer onto the tube surface. Visualization of the cells via brief exposure of the tube to blue MTS solution proved its full coverage with viable cells, as indicated by the dark blue color of the cells (figure 5(C)). Furthermore, fluorescence microscopy of cryo-cuts of the cell-covered tube revealed a thin, continuous cell layer on the tube surface (figure 5(D)).

3.3. Vascular network formation in a statically cultured 3D collagen construct

Before combining the cell sheet-covered vascular vessel mimic with self-organizing single HUVECs, we studied the network formation capacity of collagen-embedded HUVECs in a membrane insert under

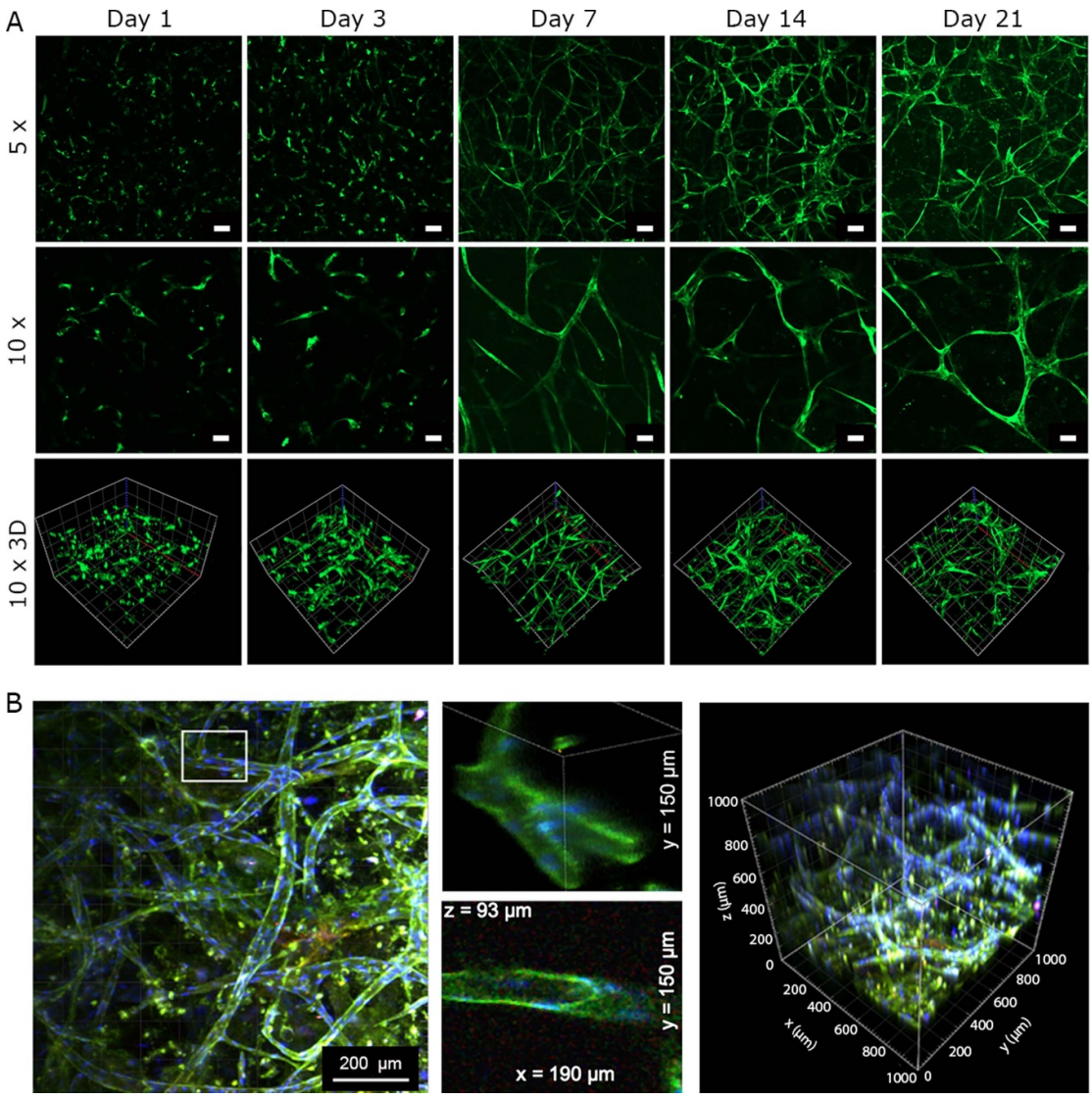

Figure 6. (A) Representative confocal microscope images revealed the vascular-like network formation of GFP-HUVECs (green) in the collagen matrix, illustrated by single z-layers (5 μm) of the fixed hydrogels (5× and 10×) and by z-stacks of the whole hydrogels (10× 3D). The scale bar equals 100 μm (5×) and 50 μm (10×). (B) Two-photon microscope images of the GFP-HUVEC network (green) on day 21, revealing a hollow lumen of the formed vascular structures; excitation wavelengths were 850 nm and 920 nm for GFP (green cells) and Hoechst (blue nuclei), respectively.

indirect coculture conditions with HDFs. Previously, the presence of HDFs outside the insert was proved to maintain the long-lived vascular-like network structure of HUVECs seeded in 2D between two collagen layers [27]. Here, we studied the vascular network formation of suspended GFP-HUVECs encapsulated homogenously within a 3D collagen gel. As seen in the fluorescent confocal microscopy images (figure 6(A)), the cells started self-organizing into a network soon after seeding, with the first morphological indication on day 3. After vasculogenesis, i.e. *de novo* formation of a primitive HUVEC network, the signs of angiogenesis were detected as sprouting from the existing vasculature. Within the first week, an interconnected HUVEC network was formed, and during the full 3 week culture period, this vascular-like structure first became denser and finally developed into a more defined network with longer tubular structures and less thick interconnections. Without HDFs, no formation of a stable HUVEC network was detected (data not shown). The stiffness of the collagen hydrogel was measured by studying its storage modulus with an oscillating rheometer at a 0.5 Hz frequency and a 0.5% strain. A collagen concentration of 2.5 mg ml^{-1} yielded hydrogels with the storage modulus of 246 $\pm$ 4 Pa (figure S4), the hydrogels being soft enough to allow efficient HUVEC migration and vascular-like network formation while strong enough to allow their handling without disruption. The resulting vasculature sprouted throughout the 3D collagen hydrogel and maintained its stability until the end of the 3 week study. Two-photon microscopy of the Hoechst-stained cells on day 21 allowed closer imaging of the network (figure 6(B)), revealing a hollow lumen structure in this network that expanded up to 1 mm in the z-range.

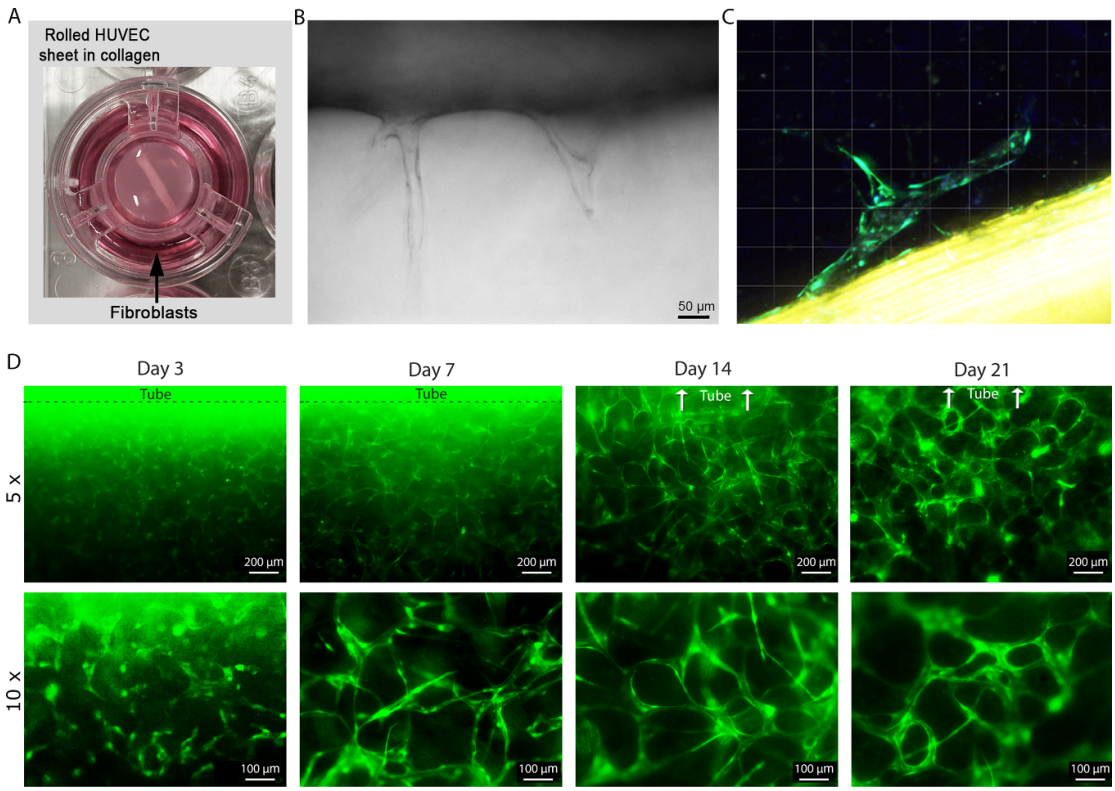

Figure 7. (A) GFP-HUVEC-covered tubular scaffold embedded in a collagen matrix in a membrane insert; (B) brightfield and (C) two-photon fluorescence microscopy image of sprouting cells from the rolled sheet (GFP in green, Hoechst in blue). (D) Representative fluorescence microscopy images of the collagen gels comprising the tube and suspended single GFP-HUVECs (green), revealing vascular-like network formation around the central vessel-mimicking tube.

3.4. Vascular sprouting from the rolled sheet and combined vascularization from the tube and the surrounding cells

After proving the vascularization capacity of collagen-embedded single HUVECs under indirect co-culture conditions, we embedded the cell sheet-covered tube into a collagen gel. First, we studied the sprouting capacity of rolled HUVEC sheets in collagen hydrogels without singularized HUVECs under indirect co-culture with HDFs (figure 7(A)). Using brightfield microscopy, we detected vascular sprouts protruding from the tube into the collagen matrix (figure 7(B)), whereas two-photon microscopy enabled a closer look at sprouting, revealing a thin cell layer on the tube surface and a bifurcating vascular sprout starting from this layer (figure 7(C)). Encouraged by the sprouting capacity of the GFP-HUVEC sheet, we embedded the rolled cell sheet in a GFP-HUVEC-laden collagen gel. Network formation around the tube under indirect co-culture was imaged using fluorescence microscopy (figure 7(D)). Although the vicinity of the vessel-mimicking tube could not be zoomed in because of its bright fluorescence, the images showed vascular-like network formation within the gel in a similar manner to the hydrogels without an embedded tube. An interconnected vascular-like network was detected throughout the collagen gel after 7 days, and the network matured and became stronger within the full 3 week culture period.

3.5. Dynamic perfusion of the collagen constructs

After establishing the suitable conditions for 3D vascularization in a static insert culture, vascularization in a physiologically more relevant dynamic perfusion culture was demonstrated. By connecting two separate cell culture chambers in a row in one medium flow circuit, we were able to culture HUVECs at a longer spatial distance from the HDFs compared to the static culture. Furthermore, the tube chamber was designed to allow real-time visualization of the hydrogel-embedded cells through the transparent top and bottom lids (figure 8(A)). Similar to the static experiments in membrane inserts, the cell sheet-covered tube was placed on top of an acellular collagen gel in the chamber together with the cell-laden collagen solution around the tube (figure 8(B)). The hydrogel-containing tube chamber was connected to the first perfusion pump to provide constant medium flow through the cell-covered central tube as well as to the second perfusion pump (0.7 ml min^{-1} for both pumps) to provide constant medium flow on top of the collagen gel (figure 8(C)). As the presence of HDFs was necessary to maintain the vascular-like network, our new dynamic culture system enabled continuous conditioning of

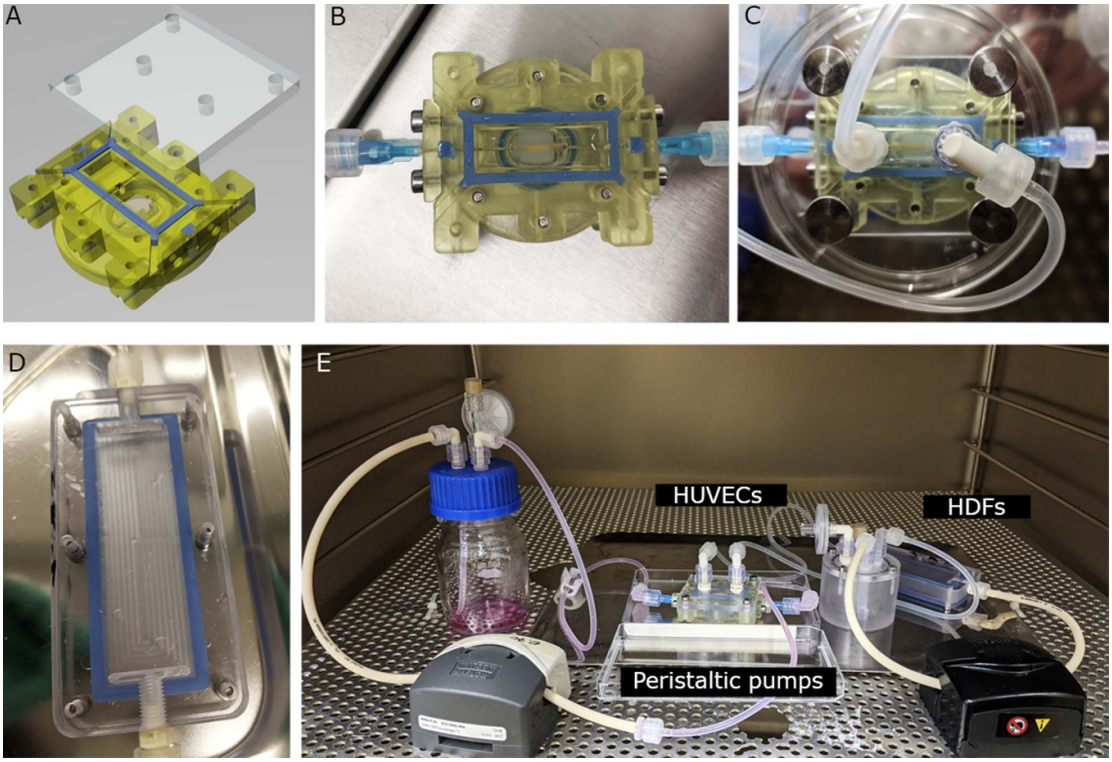

Figure 8. (A) CAD model of the tube chamber and (B) photograph of the 3D-printed chamber filled with HUVEC-laden collagen gel and a HUVEC sheet-covered tube connected to medium perfusion. (C) The hydrogel-containing tube chamber was further covered with a transparent plastic lid and connected to the second medium flow circuit from above, additionally passing through the HDF-containing slide chamber to condition the medium with proangiogenic factors. (D) Photograph of the slide chamber hosting the HDFs and (E) the whole dynamic culture system comprising the HUVEC-hosting chamber connected to two independently addressable circuits, one connected to the tube inside the chamber (red DMEM medium) and one connected to the top of the chamber (transparent conditioned HUVEC medium via passing through the HDF slide chamber).

the HUVEC medium with HDFs by perfusing the medium through the slide chamber before reaching the collagen gel (figures 8(D) and (E)). Bright-field microscopy imaging revealed no difference in the morphology and density of the HDFs cultured in DMEM, as in the static culture, versus in the 0.5% FBS Vasculife® VEGF medium used in the dynamic circuit (figure S5). Only slow diffusion of the red medium from inside the cell-covered central tube to the surrounding gel was detected, indicating at the same time the desired porosity of the tube wall and the absence of leakage in the system.

CDF simulation was used to confirm the desired laminar medium flow both on top of the collagen hydrogel and in the central tube. In the top circuit (figure 9(A)), the average flow velocity above the hydrogel was 0.029 cm s^{-1} and the average surface shear stress was 0.0034 dyn cm^{-2}. In the cell sheet-covered tube (figure 9(B)), the average and maximum medium velocity were 5.9 cm s^{-1} and 11.6 cm s^{-1}, respectively, and the average surface shear stress inside the tube was 8.9 dyn cm^{-2}.

In the dynamic culture, the HUVEC-laden collagen gel (2×10^6 cells ml^{-1}, the initial height of 2 mm) was maintained for one week under the constant flow (0.7 ml min^{-1}) of the HDF-conditioned medium. Some visible shrinkage of the collagen gel was observed both in the dynamic and static cultures, which was likely due to the traction forces applied by the networking HUVECs as well as cell-mediated hydrogel remodeling. Whereas in the absence of HDFs, HUVECs did not form any stable network in our current dynamic culture setup (data not shown), the 7 day dynamic cell culture under indirect co-culture with HDFs resulted in an interconnected vascular-like network similar to the static control (figure 10(A)) as long as the initial hydrogel thickness in this setup was around 2 mm (figure S6). In the dynamic culture, the HDFs formed a visibly thicker layer on the cell culture slide compared to the static culture, while the live/dead staining of the HDFs at the endpoint revealed high cell viability in both dynamic and static culture (figure 10(B)) FACS analysis showed that the cell viability was only slightly reduced in the dynamic culture compared to the static culture (figure S7), indicating that the indirect co-culture of HDFs with HUVECs under the dynamic medium flow did not severely interfere with their viability. The ELISA growth factor assay of the cell culture medium on day 7 revealed a higher VEGF content in the dynamic cell culture circuit than in the static culture inside the insert, even though the increased VEGF content required the presence of both HDFs and HUVECs in the circuit. The absence of one

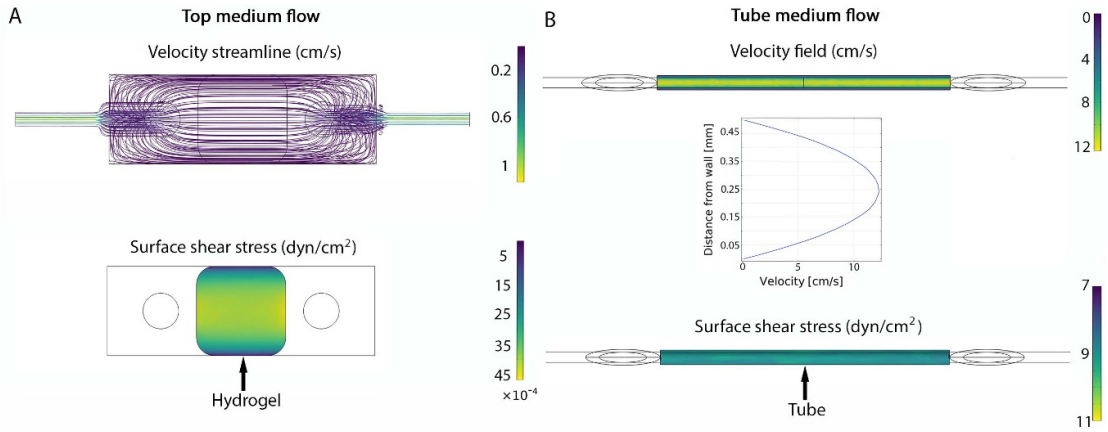

Figure 9. Medium velocity profile and the resulting surface shear stress estimated with CFD simulation (A) in the top medium circuit and (B) in the tube medium circuit.

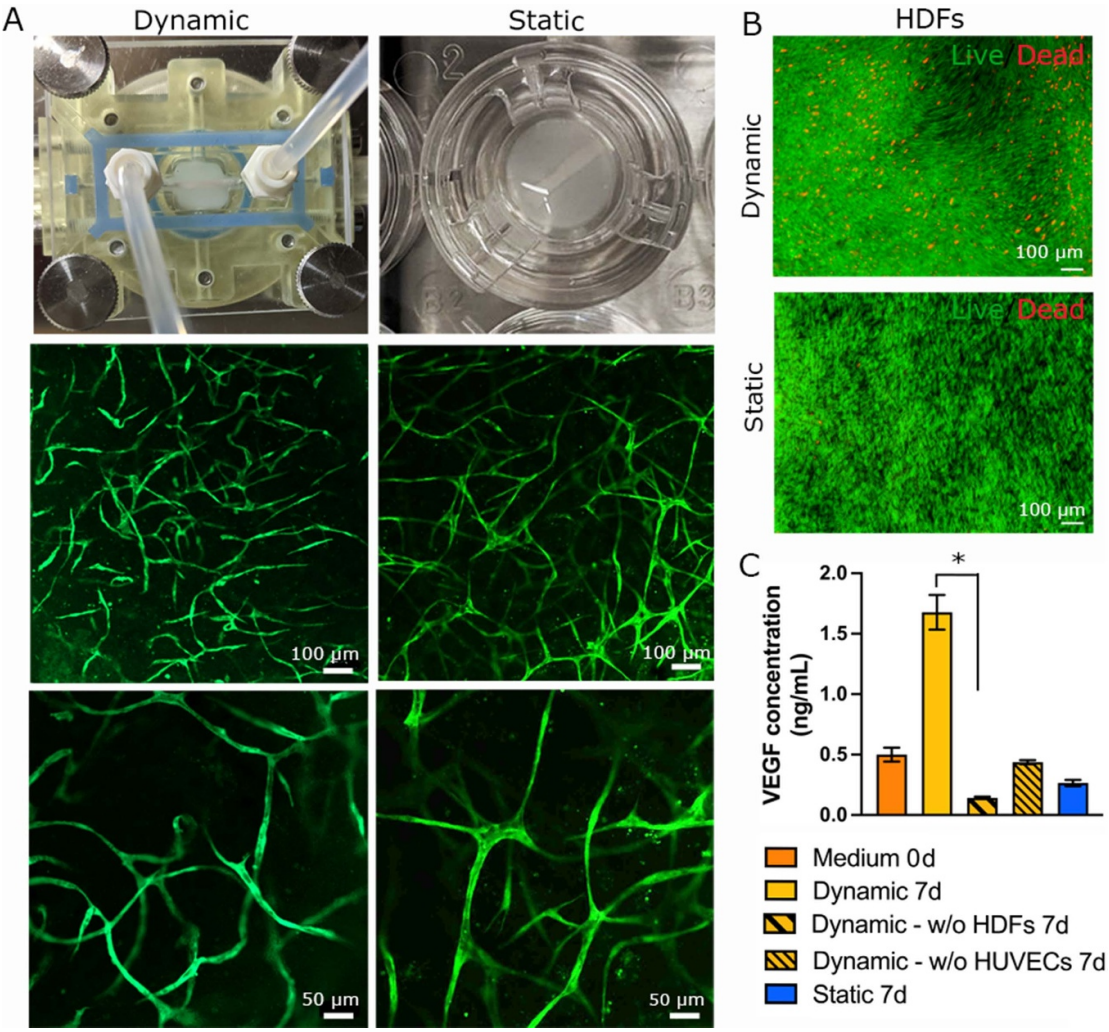

Figure 10. (A) Representative fluorescence confocal microscopy images of the HUVEC-laden collagen gels, revealing the formation of an interconnected vascular-like network within a 7 day culture in the dynamic tube chamber similar to a static insert control cultured in HUVEC medium (CMFDA-stained HUVECs in green). (B) Fluorescence microscopy images of the live/dead-stained HDFs (green/red, respectively), showing their full confluency and a high viability after the 7 day culture under both dynamic and static conditions. (C) The amount of VEGF in the cell culture medium, showing a highly increased amount in the dynamic culture on day 7 when both the HUVECs and HDFs were present in the circuit (error bar represents SD; $n = 3$; *indicates statistical significance ($p < 0.05$)).

of them led to lower VEGF concentrations resembling the one detected in VEGF-supplemented media at the beginning of the study on day 0 (figure 10(C)).

4. Discussion

The formation of an interconnected 3D vascular network has been a long-standing challenge in tissue engineering, and owing to its critical role in developing *in vitro* tissue constructs, new approaches are urgently needed. In our vascularization strategy, we simultaneously relied on 3D printing, cell sheet engineering, and self-organization of endothelial cells into a vascular-like network under proangiogenic conditions. First, to provide a 3D collagen hydrogel with a central, perfusable blood vessel mimic to aid vascularization, we rolled a confluent HUVEC sheet around a small-diameter (1.5 mm) tubular scaffold that was 3D printed of a GelMA/PCL-MA hybrid resin with a DLP printer. The GelMA-containing resin was previously developed and optimized for vat photopolymerization to enable fast and reproducible fabrication of flexible, nutrient-permeable, and biodegradable scaffolds that support cell adhesion and proliferation. The reactive blending of GelMA resin with PCL-MA enhanced the stability of the material, making it durable enough for rolling the cell sheets and long-term medium perfusion. By matching the inner diameter of the 3D-printed tubular scaffold (0.5 mm) to tightly fit the needle diameter (0.55 mm), we ensured a leakage-free connection of the tube to the perfusion chamber via the syringe needles. The adjustable biodegradation of the hybrid GelMA/PCL-MA material shown previously in an accelerated biodegradation study [24] allows the gradual loss of the mechanical support from the tube when the vascular vessel matures. In the establishing phase of the current work, the long-term stability (>3 weeks) of the material in the cell culture was desired to provide the rolled cell sheets with prolonged support.

To fabricate the cell sheets, we used PS slides covered with a thin layer (∼5 nm) of a thermoresponsive PGE polymer, as previously described [23]. Because of the carefully designed phase transition temperature in aqueous media and the fine-tuned surface coating parameters, the polymer coating remained hydrophobic and highly cell adhesive as long as it was maintained in the cell culture incubator at 37 °C, while the partial cell sheet detachment could be detected after 10 min after transferring the slide to PBS at RT. Instead of using commercially available poly(*N*-isopropylacrylamide)-coated thermoresponsive cell culture dishes, such as Upcell® plates, the application of PGE coating enabled the geometry-independent fabrication of custom-size thermoresponsive slides for preparing cell sheets. Notably, these PGE-coated slides did not require additional coating with cell adhesion promoters to allow or improve the attachment and growth of HUVECs. To enable an easy and efficient transfer of the cell sheet, we used a custom-made device for rolling and translocating the sheet to the outer surface of a tubular GelMA/PCL-MA-based scaffold. The wall-less slide geometry of the thermoresponsive cell culture substrate was required to allow its placement underneath the manually driven, rotating scaffold, ensuring pressure-free contact between the cell sheet and the scaffold, and manual adjustment of the rotation speed of the tube (video S1). Compared to the manual wrapping techniques reported elsewhere [20, 22], the rolling device enabled the fast and reliable transfer of the sheet as soon as the edges of the sheet had detached, resulting in a homogeneous HUVEC-containing layer around the tube; the hybrid material used for the 3D printing of the tube ensured efficient and sustained adhesion between the scaffold and the transferred cells.

For the sake of potential long-term maturation of our bioengineered vessel mimics, it was important to preserve the high viability and sprouting capacity of HUVECs in the rolled cell sheet. Fluorescent staining revealed very few dead cells in detached HUVEC sheets (figure 5(B)), indicating that the thermally triggered, non-enzymatic detachment did not negatively impact the cell viability, as also previously reported [23, 28]. Importantly, bifurcating sprouts were detected in brightfield and fluorescent images to protrude from the HUVEC-covered tube when embedded in collagen in indirect co-culture with HDFs (figure 7). Similar microvascular sprouting has been previously detected to start from an electrochemically deposited HUVEC layer in a collagen-based scaffold, as well as from a HUVEC layer growing on dextran-coated beads in fibrin gels [29]. However, we showed for the first time that HUVEC sheets cultured on thermoresponsive substrates and non-enzymatically detached by a thermal trigger can retain their sprouting capacity. This makes cell sheet engineering extremely useful for *in vitro* vascularization approaches. As with a cell sheet, a confluent monolayer of endothelial cells can be deposited at once in a highly defined manner into a 3D hydrogel. However, as we did not expect sprouting from the cell-covered tube to vascularize the entire 3D collagen gel, we combined it with self-organizing suspended HUVECs to form a dense vascular bed around the tube in the hydrogel. In the indirect co-culture with HDFs, we obtained a dense, lumen-containing vascular-like network that maintained its interconnected structure during the whole 3 week cell culture period. The fluorescence microscopy imaging of our vascular-like networks indicated the presence of lumenized and bifurcating vascular sprouts, which are the main characteristics of a native vasculature. Based on the microscopy images, the diameter of the vascular sprouts ranged between 25 μm and 70 μm which is in the physiological range of

human arterioles [30]. However, as we did not incorporate smooth muscle cells that natively surround arterioles, the engineered vasculature more closely resembled capillaries that consist of only endothelial cells, even though the diameter of native capillaries is smaller, ranging from 9 to 12 μm [30]. As the limit of oxygen diffusion in tissue is considered to be 100 μm, the distance between two capillaries should not exceed 200 μm [31, 32]. On the basis of the confocal microscopy images, the vascular-like network in our collagen hydrogels met this criterion. As expected from previous studies [27, 33], no stable HUVEC network was formed in the absence of HDFs. This was presumably related to the rapid loss in activity of the unbound medium-supplemented VEGF and fibroblast growth factor (FGF) as they are known to be unstable in the presence of cells [34]. Therefore, the HDFs were needed to constantly supplement the medium with proangiogenic growth factors, especially VEGF and FGF, which are known to be essential for *in vitro* formation of a lumenized vascular network [35]. In contrast to previously published 3D HUVEC networks that were obtained in a direct co-culture approach with human adipose tissue-derived stromal cells [36, 37], the HUVEC network formation in our study was purely supported by molecular cross-talk with HDFs, thereby lacking any direct cell-cell contacts between the two cell types that could interfere with each other's growth. The resulting vascular-like network expanded throughout the hydrogel (figure 6).

To enable physiologically relevant dynamic medium flow within the vascularizing collagen hydrogels, we CAD-modeled and 3D printed an autoclavable perfusion chamber that can be connected to two independent medium flows using peristaltic pumps: one providing medium through the tubular cell sheet-covered scaffold to mimic blood stream and another HDF-conditioned medium to the top surrounding of the collagen gel to ensure the constant presence of proangiogenic growth factors in the circuit. When setting up the dynamic culture system, 3D printing enabled us to fabricate a hollow scaffold for the central vascular mimic, as well as to rapidly prototype the CAD-modeled, adjustable cell culture chambers. Furthermore, using the Arduino microcontroller, we were able to simultaneously control several peristaltic pumps at the same time. The speed of the perfusion pumps was selected so that the flow velocity and the resulting surface shear stress inside the central vascular tube were close to the physiological range. The CFD simulation confirmed that the average flow velocity inside the tube was 11.6 cm s^{-1}, which was in the physiological range of arteries (approximately 3–23 cm s^{-1}), and the average surface shear stress was 8.9 dyn cm^{-2}, which was near the lower limit of an arterial surface shear stress (approximately 10–70 dyn cm^{-2}) [30]. On top of the collagen hydrogel, the low flow velocity was intended to enable cellular cross-talk between HUVECs and HDFs without no significant shear stress to the cells (0.0034 dyn cm^{-2}).

In contrast to the static insert culture, where HUVECs and HDFs were located in direct proximity, although membrane-separated, in the dynamic circuit, the cells were cultured in different chambers. Despite the increased distance between these two cell types, an interconnected vascular-like network formed similar to the static culture within a 7 day dynamic culture period, as long as the initial thickness of the HUVEC-laden collagen gel in our current cell culture setup was around 2 mm (figure S6). This indicated flexibility in the distance between the HUVECs and HDFs in their indirect co-culture, thereby enabling more versatile cell culture setups compared to static cell culture inserts, especially when the physical proximity of HDFs and network-forming HUVECs is not desired or practical. To confirm the quality of the conditioned medium in this setup, we used an ELISA growth factor assay to study the VEGF content of the medium after the 7 day cell culture, revealing a strong increase in its amount under the applied dynamic flow compared to the static culture conditions. This was primarily attributed to the higher surface area of the confluent HDF layer in dynamic culture (19 cm^2 instead of 3.8 cm^2). However, within the one-week cell culture, the higher VEGF content did not increase the density of the HUVEC network compared to the static conditions, indicating that VEGF among the myriad HDF-derived growth factors is not the limiting factor for the observed network formation. In our current proof-of-concept study, we did not focus on the long-term maturation of the vasculature in dynamic culture; therefore, the anastomosis of the cell-covered central tube into the surrounding vascular network remains to be studied in detail. However, by combining these two elements in one system with the help of cell sheet engineering and 3D-printed scaffolds and perfusion chambers, we took an important step toward the long-term goal of a perfusable vascular network that could provide cells with nutrients for a prolonged time within a bioengineered 3D tissue construct.

5. Conclusions

We successfully vascularized 3D hydrogel constructs by combining a rolled HUVEC sheet as a central vessel mimic with the surrounding self-assembling HUVECs in a collagen matrix. In the resulting hydrogels indirectly co-cultured with HDFs, the HUVECs were organized into an interconnected vascular-like network throughout the 3D matrix, while the cell sheet-covered tube sprouted to the vascularized gel. The central vascular vessel, with its preserved sprouting capacity, provided defined access ports to enable the connection of the hydrogel construct to a dynamic

medium flow. Two independently addressable and fully dynamic medium flow circuits supported the formation of an interconnected 3D vascular-like network in collagen gel, even when the proximity of HDFs in static culture conditions was replaced by their remote location under dynamic conditions. As all parts in our dynamic circuit system are easily available at relatively low costs, we hope to help readers gain access to dynamic cell culture circuits without the need for high-cost investments in bioreactors. Our achievements in this study pave the way for future bioengineered vascularized tissue constructs that could ideally provide cells with nutrients for an extended time.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments

The authors thank German Federal Ministry of Education and Research (FKZ: 13N13523; M W, L E), Dahlem Research School and the Focus Area 'Nanoscale' at Freie Universität Berlin (L E), and the Helmholtz Graduate School for Macromolecular Bioscience (M L, M W) for the financial support of the current work as well as the Core Facility BioSupraMol supported by Deutsche Forschungsgemeinschaft for the use of SEM facilities. Furthermore, Dr Daniel Stöbener is thanked for providing the PGE polymer for the thermoresponsive coatings of the cell culture slides. M W is grateful to constant support from the institute of pharmacy (Professor B Kleuser, Professor M Schäfer-Korting and Professor S Hedtrich) at Freie Universität Berlin throughout this project.

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