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RESEARCH ARTICLE

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# Porous collagen scaffolds enable endothelial lumen formation in vitro under both static and dynamic growth conditions

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## Abstract

Despite recent advances in the field of tissue engineering, the development of complex tissue-like structures in vitro is compromised by the lack of integration of a functioning vasculature. In this study, we propose a mesoscale three-dimensional (3D) in vitro vascularized connective tissue model and demonstrate its feasibility to prompt the self-assembly of endothelial cells into vessel-like structures. Moreover, we investigate the effect of perfusion on the organization of the cells. For this purpose, primary endothelial cells (HUVECs) and a cell line of human foreskin fibroblasts are cultivated in ECM-like matrices made up of freeze-dried collagen scaffolds permeated with collagen type I hydrogel. A tailored bioreactor is designed to investigate the effect of perfusion on self-organization of HUVECs. Immunofluorescent staining, two-photon microscopy, second-harmonic generation imaging, and scanning electron microscopy are applied to visualize the spatial arrangement of the cells. The analyses reveal the formation of hollow, vessel-like structures of HUVECs in hydrogel-permeated collagen scaffolds under both static and dynamic conditions. In conclusion, we demonstrate the feasibility of a 3D porous collagen scaffolding system that enables and maintains the self-organization of HUVECs into vessel-like structures independent of a dynamic flow.

KEYWORDS

bioreactor, collagen, dynamic culture, endothelial cell, pre-vascularization

## 1 | INTRODUCTION

In medical treatments, the substitution of tissues using grafts is often restricted by finite availability as well as the generation of new defects at donor sites.[1,2] Similarly, the reconstruction of complex three-dimensional (3D) models, used for the research of physiological processes[3–6] and pathological conditions is still a challenge.[3,5,6] With the aim of developing tissues to stand in for their autologous equivalents or function as preclinical 3D models in pharmacological research, tissue engineering presents a promising alternative to tissue grafting.[7,8] Despite recent advances in the field of regenerative medicine, however, the complexity of the developed tissues remains limited. One limitation is the fact that the diffusion of nutrients and gases in human tissue is confined to a distance of about 200 µm,[9] and the implementation of a functioning vasculature remains challenging. Lack of vessels in turn impedes the supply of oxygen and nutrients to cells, and hinders the removal of waste products. Consequently, the cells experience hypoxia, eventually resulting in the destruction of the newly developed tissue.[2,9,10] By providing a functioning vasculature, the development

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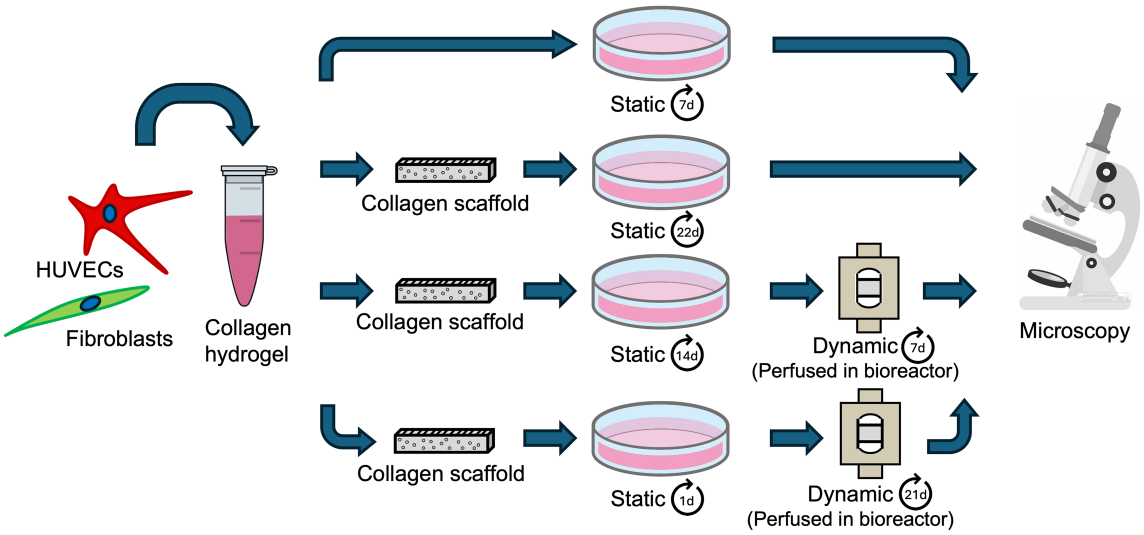



**FIGURE 1** Schematic representation of the different culturing conditions. Primary endothelial cells and a cell line of human fibroblasts were suspended in collagen hydrogel. Four different conditions were investigated: (1) The arrangement of the cells in collagen hydrogel alone under static conditions, (2) the effect of static cultivation within hydrogel-infused freeze-dried scaffolds for 22 days, (3) the effect of dynamic cultivation within hydrogel-infused freeze-dried scaffolds inside a perfusion bioreactor following a cultivation under static conditions for 14 days, and (4) the effect of dynamic cultivation for 22 days within hydrogel-infused freeze-dried scaffolds inside a perfusion bioreactor after only 1 day of static cultivation.

of complex tissue equivalents with an increased functionality can be facilitated.

Different methods such as proangiogenic and prevascularization approaches have been attempted with the goal of introducing a vascular network into 3D in vitro tissue models.[11,12] However, as the formation of new blood vessels via angiogenesis is usually slow this approach may not always be feasible in tissue engineering.[12–14] Accordingly, particular attention has been devoted to developing effective prevascularization methods through various techniques including 3D (bio) printing.[15–18] This technique utilizes sacrificial hydrogels to create hollow structures that can be populated with endothelial cells in a subsequent step.[19–21] However, this method requires access to a bioprinter and involves technically complex procedures. Additionally, the resolution that is currently being achieved by bioprinting is considered relatively low. Moreover, depending on the method, the cells are exposed to bioprinting-associated mechanical and thermal stress.[22–25]

A possible alternative to (bio)printing is offered by targeting the self-assembly of endothelial cells into vessel-like structures.[26] With this method, the self-organization of the endothelial cells can be initiated via signaling molecules such as integrins[6] or proangiogenic factors such as vascular endothelial growth factors, angiopoietins, and platelet-derived growth factor-BB.[4] Similarly, the endothelial cells' microenvironment, including the composition of the extracellular matrix (ECM) and its stiffness, is known to be a determining factor in the function of endothelial cells and hence the formation of capillary-like structures in vitro.[27–29] Additionally, it has been shown that mechanical cues such as fluid shear stress can enhance the migration of endothelial cells[29] and that the interplay between fluid shear stress and matrix stiffness affects endothelial cell function.[30]

In this study, we hypothesized that the use of a porous, freeze-dried collagen scaffold provides geometrical and chemical cues to prompt the self-assembly of endothelial cells into vessel-like structures in vitro. For this purpose, primary endothelial cells were loaded in collagen hydrogel and infused into a 3D collagen-based scaffold. To increase the complexity of the system and more closely approach in vivo-like conditions, human foreskin fibroblasts were introduced for ECM production and to provide a necessary biophysical and biochemical support to the endothelial cells. Our 3D model was then cultured under either static and/or dynamic growth conditions. Figure 1 schematically depicts the different culturing conditions examined in this work. In the case of dynamic cultivation, the cell-laden collagen scaffold was perfused in a custom-made perfusion bioreactor to investigate whether the effect of flow exposure enhances the self-organization of the cells into vessel-like structures and to explore if the endothelial cells will sustain their network under perfused conditions. Two-photon microscopy, second harmonic generation imaging and scanning electron microscopy were utilized to qualitatively evaluate the success of prevascularization in our in vitro engineered tissue model.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from the *vena umbilicalis* from the human umbilical cord after informed consent approved by the Ethics Committee of RWTH Aachen University Hospital (EK 424/19). The cells were cultivated in endothelial cell

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culture medium (Endothelial Cell Basal Medium 2/Kit, EBM 2, PromoCell, Heidelberg, Germany) supplemented with penicillin–streptomycin (Penicillin–Streptomycin, 10,000 U/mL Penicillin, 10 mg/mL Streptomycin, PANBiotech, Aidenbach) and passaged at a confluency of 80% in a split ratio of 1:5. HUVECs were used until passage 5. A cell line of human foreskin fibroblasts (BJ cells) was provided by Dr. Nicole Schwarz from the Institute of Molecule and Cellular Anatomy (RWTH Aachen University Hospital, Aachen, Germany). The fibroblasts were cultured in fibroblast culture medium composed of DMEM (Dulbecco's Modified Eagle's Medium (high glucose), Sigma-Aldrich, St. Louis, MO, USA), Medium 199 (Medium 199, Sigma-Aldrich, St. Louis, MO, USA), L-glutamine (L-Glutamine Solution, 200 mM, Sigma-Aldrich, St. Louis, MO, USA), penicillin–streptomycin (Penicillin–Streptomycin, 10,000 U/mL Penicillin, 10 mg/mL Streptomycin, PANBiotech, Aidenbach) and FBS (fetal bovine serum, P30-1502, PANBiotech, Aidenbach). The cells were cultured at 37°C, 5% $CO_2$ and 90% humidity inside an incubator.

## 2.2 | Preparation of collagen hydrogels

Collagen hydrogels were prepared by mixing 0.7 M NaOH (sodium hydroxide, M = 40 g/mol, Merck, Darmstadt, Germany) with 1 M HEPES-Buffer (PANBiotech, Aidenbach, Germany) at a ratio of 1:1 to make solution A. Solution B was prepared by mixing solution A with 10-fold DMEM (Dulbecco's Minimum Essential Medium (10x), Biochrom, Berlin, Germany) according to the manufacturer's information. Finally, collagen G (Collagen G, 4 mg/mL collagen type 1 in 15 mM HCl, Merck, Darmstadt, Germany) was mixed with solution B at a ratio of 2:8 to make the collagen hydrogel.

## 2.3 | Collagen type I scaffold

Sterile macroporous freeze-dried collagen I scaffolds (Optimaix, Matricel GmbH, Herzogenrath, Germany) made from porcine collagen I were used as extracellular matrix (ECM) for the cells. To ensure reproducible sizing and optimal fit of the collagen scaffolds inside the bioreactors, the samples were cut into 1 mm thick slices using a razor blade. The collagen scaffolds were 10 mm wide × 17 mm long.

## 2.4 | Cell loading

For all trials, the cells were prepared following the protocols described in Section 2.1. Cells were counted on an automated cell counter (Countess™ II automated cell counter, Thermo Fisher, Scientific, MA, USA) and either loaded inside the collagen blend at varying cell densities ($5 \times 10^6$ cells/mL of hydrogel, $50 \times 10^6$ cells/mL of hydrogel and $100 \times 10^6$ cells/mL of hydrogel) and left to polymerize into hydrogel or were suspended inside the collagen blend, which was subsequently used to load the pre-equilibrated porous collagen type I scaffolds (Figure 1). For the investigation of the cells inside the hydrogels alone, the corresponding number of cells were resuspended in solution B, which was mixed with collagen G in a subsequent step. This cell-laden hydrogel was then pipetted into a well of a 24-well-plate and incubated for 60 min. After 1 h, 1000 µL EBM 2 was added. For the investigation using the cell-laden hydrogels in combination with porous collagen type I scaffolds, $5 \times 10^6$ HUVECs and $5 \times 10^6$ fibroblasts were suspended in 100 µL collagen hydrogel, which was then pipetted onto collagen type I scaffolds pre-wetted with EBM 2. Here, particular attention was paid to the even distribution of the cell-laden hydrogel. Following the incubation period of 60 min, EBM 2 was added to the well plate, which was then incubated for later experimental procedure.

## 2.5 | Assembly of the perfusion bioreactor and dynamic cultivation setup

A tailored perfusion bioreactor was designed and manufactured from PEEK (Figure 2A,B). Two glass coverslips (Coverslip, L 24 mm W 24 mm, Carl Roth, Karlsruhe, Germany), rubber gaskets and 10 screws were used for the complete assembly. To create a seal between the components of the bioreactor and the glass coverslips, a thin layer of sterile 35 wt % pluronic (Pluronic, F-127, BioReagent, P2443, Merck, Darmstadt, Germany) was coated on the surface of the respective parts. For the assembly of the dynamic setup, a medium reservoir was filled with 13 mL of EBM 2 and supplemented with a sterile filter (Filtropur S, 02, Sarstedt, Nümbrecht, Germany) for gas exchange. A silicone tube with an inner diameter of 2.5 mm (Tube ROTILABO Silicone, Carl Roth, Karlsruhe, Germany) was connected to the medium reservoir, led through a peristaltic pump (Embedded Dosing Pump, EZO-PMP, Atlas Scientific, Long Island City, NY, USA), and connected to a three-way valve (Discofix C-3, B. Braun, Melsungen, Germany) and then to the inlet of the bioreactor (Figure 2C,D). Finally, the outlet of the bioreactor was connected to a tube leading back into the medium reservoir.

## 2.6 | Immunofluorescent staining

Immunostainings were performed on samples fixed in 4% paraformaldehyde (PFA, Thermo Fisher, Waltham, MA, USA) for 12 min, incubated in PBS with 1% triton X-100 (Triton X-100, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 60 min, and blocked using 3% bovine serum albumin (BSA, SERVA, Heidelberg, Germany) in PBS with 0.1% triton X-100 for 120 min. Vascular-endothelial cadherin (Anti VE-cadherin antibody (F-8), dilution 1:250, Santa Cruz Biotechnology, Heidelberg, Germany) and phalloidin (Alexa Fluor 488, dilution 1:400, Millipore, Burlington, MA, USA) were diluted in 3% BSA and incubated overnight at 4°C. After removing the primary antibody solution, the samples were washed and incubated with the secondary antibody solution Alexa Fluor 594 (Goat Anti-Rabbit IgG H&L, dilution 1:400, Abcam, Cambridge, UK) for 180 min at room temperature. The secondary antibody solution was washed out with using 3% BSA in PBS three times for 10 min and DAPI (4′,6-Diamin-2-phenylindol, dilution 1:3000, Invitrogen AG, Carlsbad, CA, USA) was

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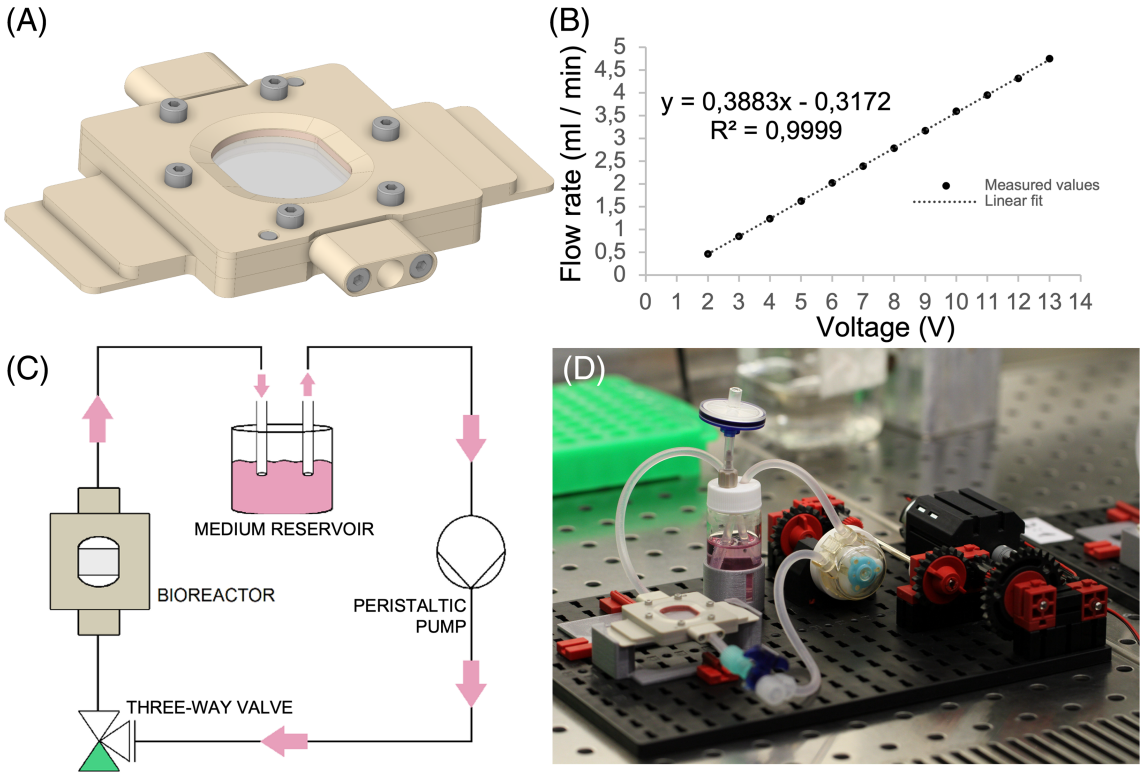



**FIGURE 2** The dynamic setup consists of a perfusion bioreactor, a peristaltic pump, a valve, and tubes to provide medium flow. (A) Top view of fully assembled perfusion bioreactor including glass coverslips, gaskets and screws (rendered image). (B) Flow rate based on the supplied voltage: The relationship between the supplied voltage and the resulting flow rate was linear. (C) Schematic presentation of the complete dynamic setup. (D) Actual image of the complete dynamic cultivation setup including the perfusion bioreactor, medium reservoir with filter, peristaltic pump, gear and a three-way valve.

incubated for 30 min. Finally, the samples were washed again with PBS three times for 10 min each.

### 2.7 | Two-Photon Microscopy and Second Harmonic Generation Imaging

The image acquisition of the 3D samples was performed using a multi-photon excitation fluorescence microscope (Olympus FV-1000-MPE Two Photon microscope with Olympus XLPN 25x WMP-SP, Na1.95 Immersion objective, Tokyo, Japan) and the image acquisition software Fluoview Fv10 2.0. The images were recorded in the X-Y-plane and collected at different Z-heights. The laser (Spectra physics MaiTai DeepSee HPDS-OL, Santa Clara, CA, USA) was operated at a wavelength of 820 nm to excite and visualize the cells. A wavelength of 840 nm was used for the excitation of the collagen scaffold using second-harmonic generation. The intensity of the laser was set between 5% and 30%.

### 2.8 | Scanning Electron Microscopy (SEM)

The microstructure of the collagen scaffolds was imaged with a 10 kV acceleration voltage in a high vacuum environment using a scanning electron microscopy (Environmental Scanning Electron Microscope, ESEM XL 30 FEG, FEI (Philips), Hillsboro, OR, USA) as described before.[31]

### 2.9 | Image preparation and analysis

The images of the cells inside the collagen scaffolds were evaluated using the open-source software *Ima geJ*.[32] In order to accentuate certain features, the z-stacks were resliced. Furthermore, the z-stacks showing the collagen scaffolds were represented as a sum of the slices, whereas the other channels were represented as the maximum intensity of the z-projection. To give an overview of the z-stacks combined, the channels were merged. To provide a view in orthogonal direction, the 3D reconstructed images were cut vertically.

## 3 | RESULTS

### 3.1 | Structure of collagen scaffold

Scanning electron microscopy revealed that the collagen scaffolds were made up of stacked sheets of collagen that were interconnected by column-like structures (Figure 3). The arrangement of the sheets

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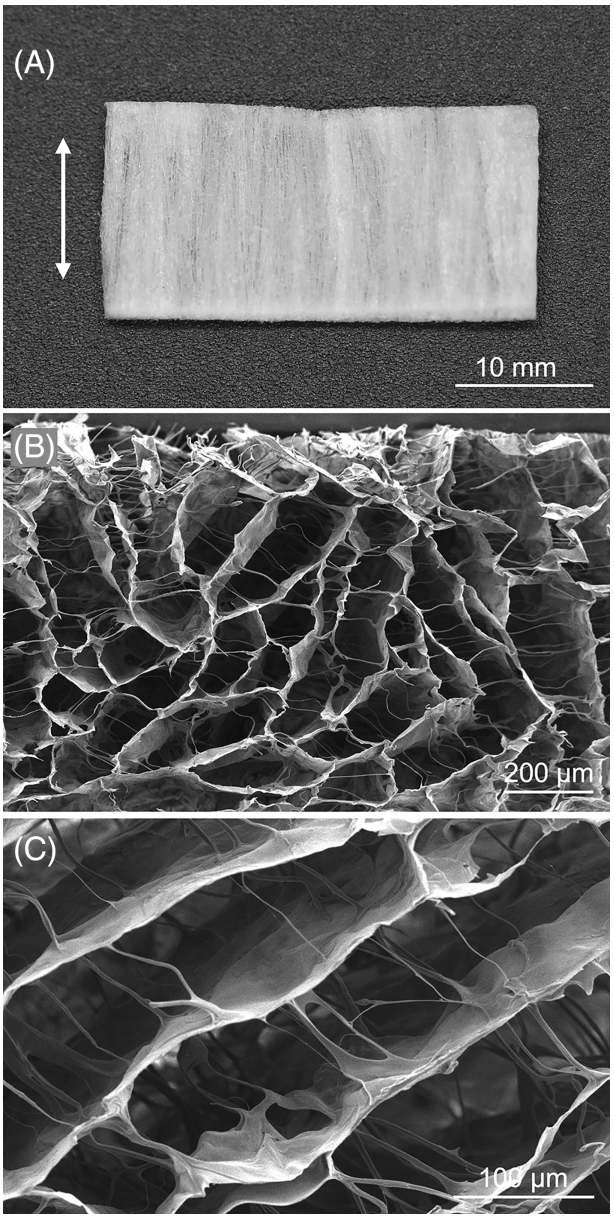



**FIGURE 3** Macroscopic and microscopic structure of porous freeze-dried collagen type I scaffolds. (A) Macroscopic top view of collagen scaffold showing the direction of the pores within the scaffold (indicated by white arrow). (B) Scanning electron micrograph showing the cross-section of the porous collagen scaffold. (C) Higher resolution of scanning electron micrograph showing the scaffolds are made up of stacked collagen sheets that are interconnected by column-like structures.

was asymmetrical and the mean distance between the individual sheets was 119.4 µm ± 34.5 µm.

## 3.2 | Cellular self-organization within a collagen hydrogel

Varying cell densities were used in a ratio of 1:1 HUVECs to fibroblasts to investigate the self-assembly of the cells within collagen hydrogels alone. At higher cell densities (100 × $10^6$ cells/mL of hydrogel) a stronger interconnection of HUVECs was observed after 7 days of cultivation. However, the formation of tubular structures could not be confirmed in any of the samples (Figure 4). Macroscopic shrinkage of the cell-laden hydrogel was observed during the experiment (not shown), which was more pronounced at higher cell density.

## 3.3 | Effect of delayed perfusion on cellular self-organization within hydrogel-permeated collagen scaffolds

For the investigation of the effects of flow and thus shear stress on the self-assembly of vessel-forming cells, HUVECs and fibroblasts were suspended in collagen hydrogels and distributed within 3D freeze-dried scaffolds as described above. Following cultivation under static conditions for 14 days, the cell-loaded scaffolds were transferred to the perfusion bioreactors in order to enable exposure to flow shear stress (Figure 5). As shown in Figure 5A, two-photon microscopy and second-harmonic generation imaging revealed that HUVECs and fibroblast cells were well integrated with the collagen scaffold prior to the introduction of flow. However, the introduction of flow and cultivation for 7 days under perfusion appeared to disrupt the integrity of some of the HUVECs within our in vitro model (Figure 5B,C), which was the case for both flow rates investigated (71 µL/min and 425 µL/min).

## 3.4 | Cellular self-organization within hydrogel-permeated collagen scaffolds under static conditions

As a result of the previous findings, the co-culture of HUVECs and human fibroblasts were cultured under static conditions for 22 days. Subsequently, two-photon microscopy revealed the formation of a continuous monolayer of HUVECs within the samples (Figure 6). Moreover, the images hinted toward the formation of a vessel-like structure at several positions within the scaffold (e.g., the yellow dashed line in Figure 6B). The 3D reconstructed image was used to provide an orthogonal view of the sample (right side of Figure 6B and indicated by arrows). The formation of a tubular structure was verified by this orthogonal view. The diameter of the described structure ranged from about 25 µm to about 70 µm and its total length was at least 420 µm. Moreover, the described structure formed by the HUVECs appeared to be hollow, because by merging the different channels no other structures (cells or scaffold) could be observed within the circumference of the structure (Figure 6D) at different cross-sectional positions. Several tubular structures of this sort were observed across the sample.

## 3.5 | Cellular self-organization within hydrogel-permeated collagen scaffolds under dynamic culture

With the purpose of investigating the effects of the immediate exposure to flow on the cells, the cultivation under static conditions for

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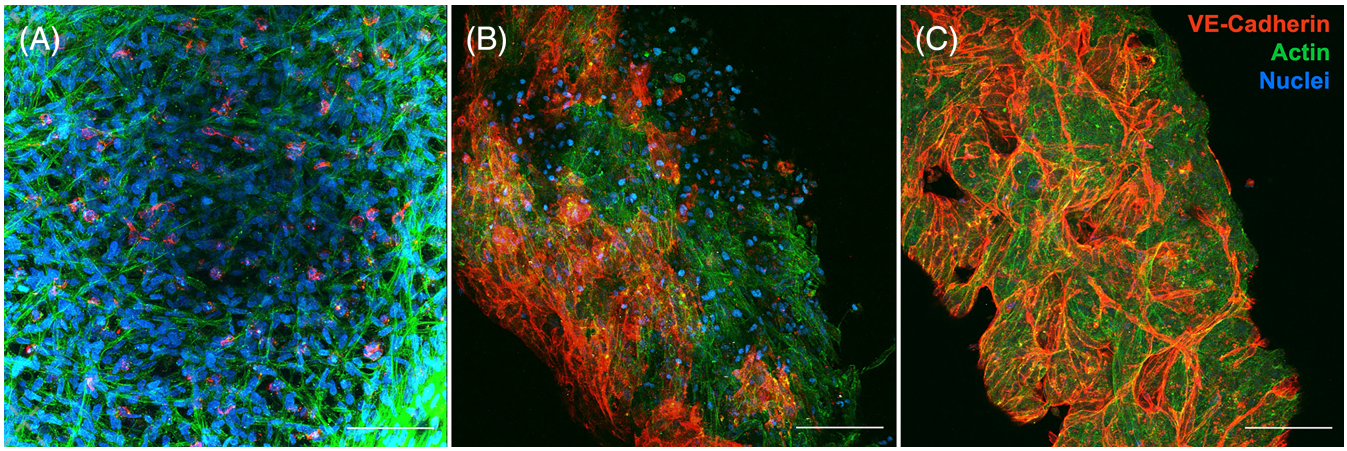



**FIGURE 4** Immunofluorescent images from a top view showing the organization of the cells in hydrogel alone at different cell seeding densities after 7 days of cultivation. (A) HUVECs and fibroblasts suspended inside a collagen hydrogel at a low seeding concentration ($5 \times 10^6$ cells/mL of hydrogel). HUVECs did not form tubular networks. (B) HUVECs and fibroblasts inside a collagen hydrogel at a medium cell seeding concentration ($50 \times 10^6$ cells/ml of hydrogel). HUVECs interconnected, but tubular structures were not observed. (C) HUVECs and fibroblasts inside collagen hydrogel at a high cell seeding concentration ($100 \times 10^6$ cells / ml of hydrogel). The HUVECs seemed to encompass the fibroblasts thereby forming a barrier around them. VE-cadherin: Red; Actin: Green; Nuclei: Blue. Scale bar (A–C): 100 μm.

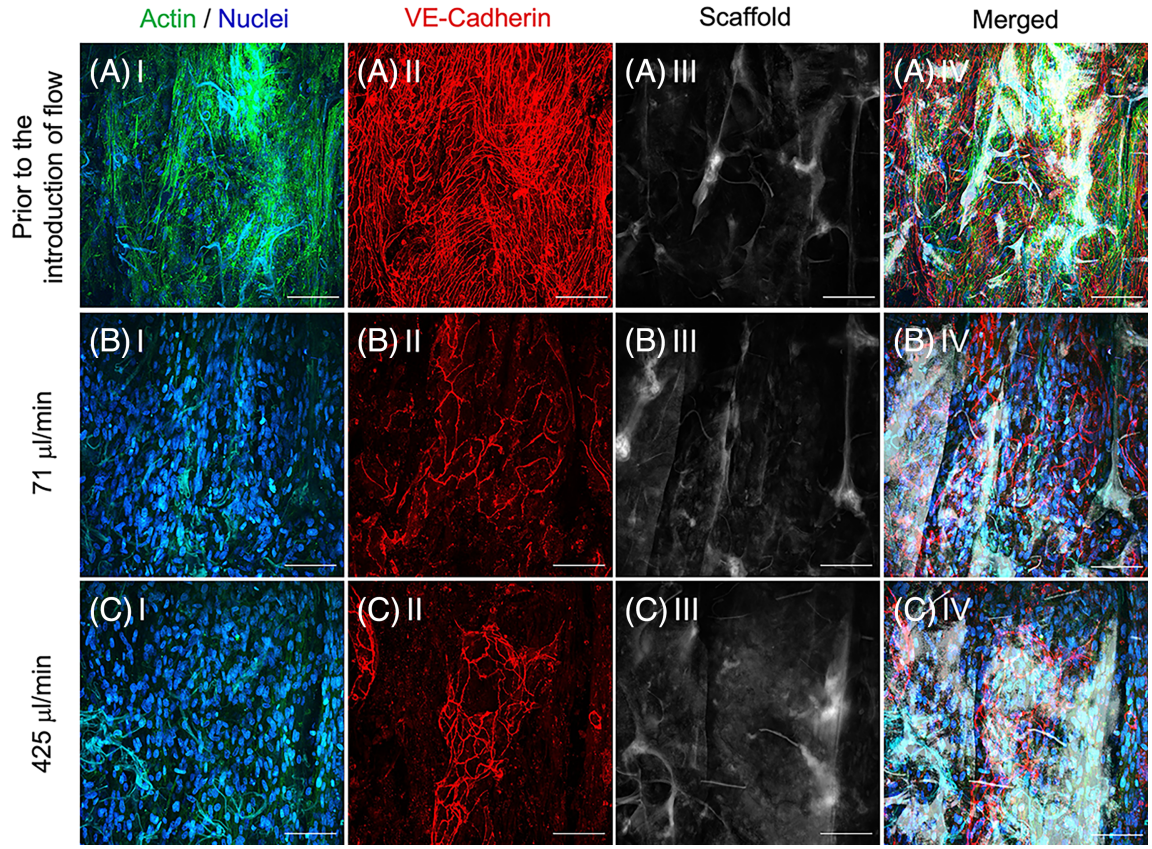



**FIGURE 5** Comparison of the network establishment in the samples that were statically cultivated for 14 days before being cultivated under different dynamic conditions for 7 days. (A) Prior to the introduction of flow, the network established by the HUVECs was congruent with the network established by all cells in the sample. (B) Overview of the sample that was cultivated under dynamic conditions at 71 μL/min for 7 days after being cultivated under static conditions for 14 days prior. (C) Overview of the sample that was cultivated under dynamic conditions at 425 μL/min. The endothelial cell (HUVEC) network appeared disrupted in this sample. VE-cadherin: Red; Actin: Green; Nuclei: Blue. Scale bars (A–C): 100 μm.

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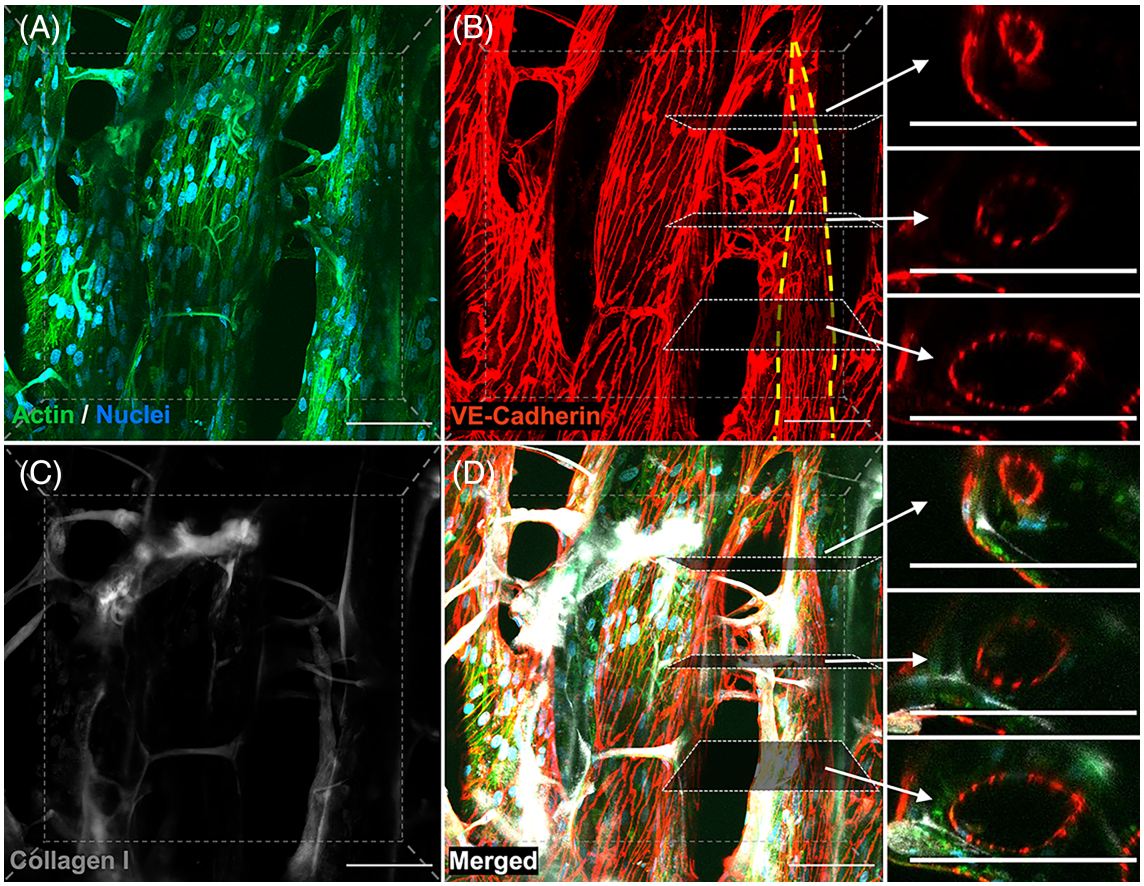



**FIGURE 6** Immunofluorescent images show the formation of tubular structures in hydrogel-permeated collagen type I scaffolds after 22 days of cultivation under static conditions. The dashed lines indicate where the cross-sectional images (insets) were obtained. The white arrows show one of the tubular structures that had formed within the sample. (A) The cells' Actin filaments are shown in green and their nuclei are shown in blue. All cells were evenly distributed across the sample. (B) The HUVECs (visualized in red) formed a continuous monolayer. Moreover, tubular structures formed by the HUVECs were observed. (C) The collagen scaffold (shown in gray) was visualized using second harmonic generation imaging microscopy. (D) The overlay of all channels shows that the tubular structure formed by the HUVECs is hollow. Scale bar (A–D): 100 µm.

24 h was followed by the transfer of the scaffold to the perfusion bioreactor, in which the cells were exposed for 21 days to a flow rate of either 71 µL/min (equivalent to an average velocity of 0.07 mm/s in scaffold cross-section) or 425 µL/min (equivalent to average velocity of 0.417 mm/s of medium in scaffold cross-section). In both cases, the cultivation resulted in the formation of a mature tissue-like structure with a continuous monolayer of HUVECs (Figures 7 and 8). Moreover, branched vessel-like structures formed by HUVECs were observed (Figures 7 and 8) and orthogonal views of the samples revealed that some of these structures were hollow. In contrast to the perfusion at a lower flow rate of 71 µL/min, the exposure to a higher flow rate of 425 µL/min resulted in a network formation of the HUVECs that was not congruent with the organization of the rest of the cells in this sample (Figure 8), demonstrated by comparing the network formed by HUVECs alone (Figure 8B) to the superimposition of all channels in the sample (Figure 8D). As visualized, in these samples the endothelial cells formed a branched structure that was independent of the scaffold and fibroblasts.

## 4 | DISCUSSION

The successful creation of a functional vasculature is a major requisite in tissue engineering of mesoscale 3D in vitro models. Without a vascular network supplying the cells with oxygen and nutrients, hypoxia and necrosis lead to the destruction of the tissue model.[2,9,10] In vivo, cells form new blood vessels during embryogenic development in a process described as vasculogenesis or form blood vessels from a pre-existing network during angiogenesis. Much effort has therefore been devoted to mimicking these processes for the formation of blood vessels in vitro. While in the past, hydrogels have commonly been used as the extracellular matrix, in this work, we focused on self-assembly of the endothelial cells using collagen scaffolds with interconnected pores that are perfusable and combined these with a collagen hydrogel. Therefore no pre-existing vessel-like structures, like those used in bioprinting, were utilized. We hypothesized that these freeze-dried open-porous collagen scaffolds prompt the self-assembly of endothelial cells into vessel-like structures in vitro and investigated the potential influence of fluid shear stress by dynamic perfusion.

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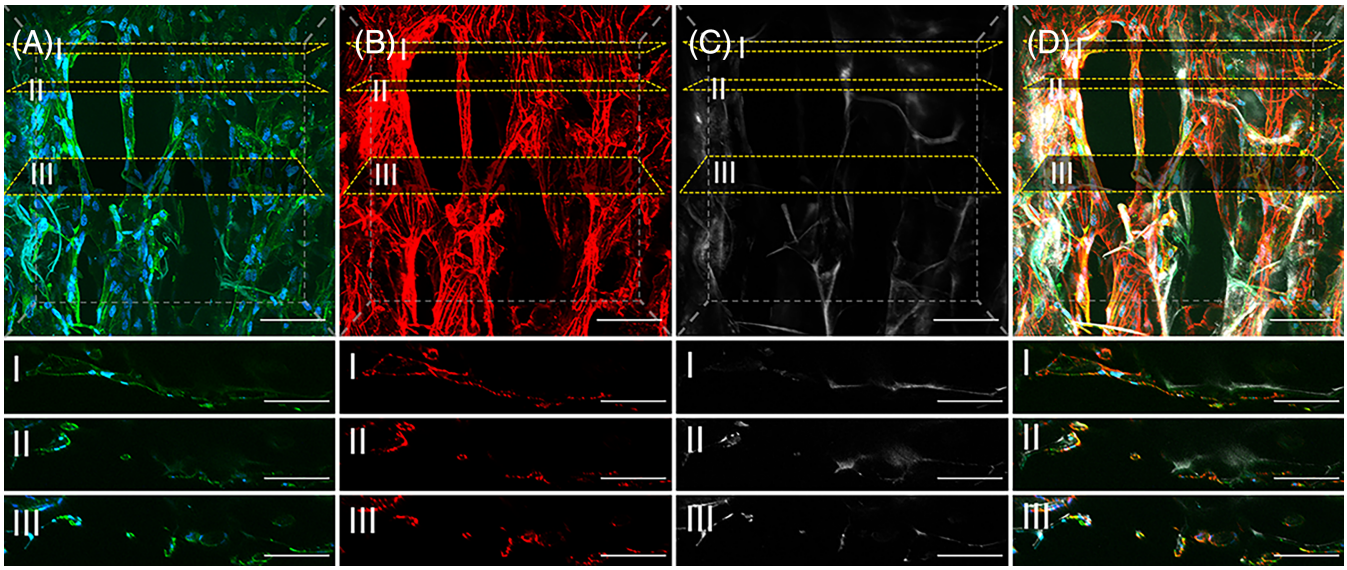



**FIGURE 7** Immunofluorescent images show the cellular organization within hydrogel-permeated collagen type I scaffolds after 21 days of cultivation under low flow rate dynamic conditions. $5 \times 10^6$ HUVECs and $5 \times 10^6$ fibroblasts were suspended in 100 µL collagen hydrogel, which was then used to permeate a porous collagen scaffold. After 24 h of cultivation under static conditions, the sample was transferred to the bioreactor and the pump was set to 71 µL/min. The cells were cultivated under these conditions for 21 days. (A) Actin filaments are shown in green and cell nuclei are shown in blue. (B) The HUVECs (represented by VE-cadherin) formed a branched tubular network. (C) The collagen scaffold (shown in gray) was visualized using second harmonic generation imaging microscopy. (D) By merging all channels, the organization of the HUVECs within the collagen scaffold was demonstrated. Cross-sectional images (insets I-III) show that some of the structures formed by the HUVECs were hollow. Scale bar (A–D): 100 µm.

A study performed under 3D conditions in 2004 suggested that endothelial cells cultured in hydrogels could be induced to self-organize into vascular-like structures by exposing the cells to flow shear stress,[33] hinting toward a proangiogenic effect of mechanical cues. Since then, research has been undertaken to investigate the effect of dynamic culture in prevascularization. For example, Salameh et al.[5] used collagen hydrogel and differently stained HUVECs to demonstrate the new formation of a secondary network of HUVECs that were located in a close proximity to but not directly exposed to flow shear stress,[5] while another publication suggested that when introduced gradually, mechanical stress may improve the morphological features of HUVEC in vitro.[34]

In order to initiate the self-assembly of endothelial cells, they require optimal culturing conditions defined by specific biochemical and biophysical conditions. These comprise an extracellular environment that enables not only the attachment but also the migration of the cells.[35] Moreover, the provision of cues to initiate the assembly of the cells into vessel-like structures should be ensured. Under physiological conditions, the extracellular matrix in most tissues is made up of collagen.[36] It exhibits RGD-motifs, which enable cellular adherence as well as cellular signaling, both of which are essential to cell migration and blood vessel formation.[37–40] In vivo, the ECM is synthesized by several cells such as ASCs and fibroblasts. Fibroblasts have been shown to enhance angiogenesis and improve the formation of capillary-like structures by remodeling the extracellular matrix and secreting pro-angiogenic factors.[28,41,42] Moreover, matrix stiffness and its composition can provide additional cues and have been described as an effective parameter in network formation of vessel forming cells.[19] Hydrogels are commonly used biomaterials as ECM substitutes in tissue engineering.[5,33,43] However, it is not possible to study the effect of flow shear stress on the embedded cells because the dense hydrogels are not perfusable. A study published in 2016 reported the formation of luminal structures by ECs in porous three-dimensional collagen scaffolds and fibrin gels after cultivation under static conditions,[44] but the effect of flow shear stress was not investigated. Therefore, we used a setup similar to the cited paper for our study. The ECM provided to the cells were freeze-dried collagen scaffolds made up of stacked asymmetrical sheets of collagen that were interconnected by column-like structures.

We commenced by developing a dynamic cultivation setup to investigate the effects of a delayed application of flow shear stress on the self-assembly of HUVEC in vitro. In this case, the cell-loaded scaffolds were cultured for 14 days under static conditions followed by perfusion inside a bioreactor for 7 days. The fluorescent images of the cell-loaded scaffolds prior to the perfusion inside the bioreactor demonstrated a cellular integrity of both cell types. Surprisingly, the introduction of flow for 7 days appeared to disrupt the integrity of some of the HUVECs within our model as observed by VE-cadherin investigation. Accordingly, we were not able to demonstrate the formation of a tubular network under these conditions. This was the case for both flow rates explored in our work. These results are in contrast to investigations, in which hydrogels had been used as ECMs.[5,33,43] These differences might be explained by the fact that the porous scaffolds do not offer enough mechanical support to the cells compared

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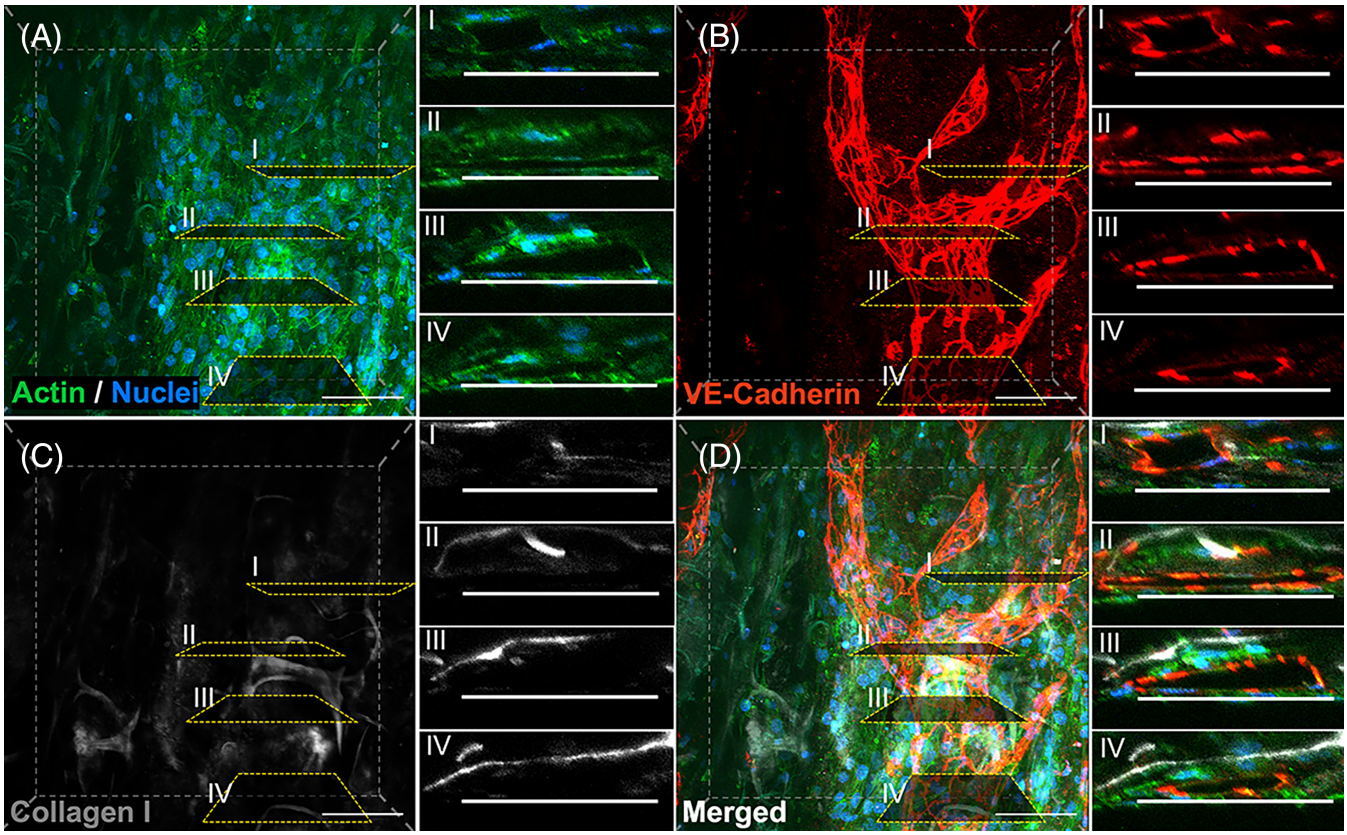



**FIGURE 8** Immunofluorescent images show the cellular organization within hydrogel-permeated collagen type I scaffolds after 21 days of cultivation under high flow rate dynamic conditions. $5 \times 10^6$ HUVECs and $5 \times 10^6$ fibroblasts were suspended in 100 µL collagen hydrogel, which was then used to permeate a porous collagen scaffold. After 24 h of cultivation under static conditions, the sample was transferred to the bioreactor and the pump was set to 425 µL/min. The cells were cultivated under these conditions for 21 days. (A) Actin filaments are shown in green and cell nuclei are shown in blue. (B) The HUVECs (represented by VE-cadherin) formed a branched tubular network. (C) The collagen scaffold (shown in gray) was visualized using second harmonic generation imaging microscopy. (D) By merging all channels, the formation of a tubular structure formed by HUVECs alone was demonstrated. Cross-sectional images (insets) show that the structure formed by the HUVECs was hollow. Scale bar (A–D): 100 µm.

to the polymetric network of hydrogels. Nevertheless, these findings initiated the comparison between the network formed by endothelial cells under static conditions and the network formed as a result of the immediate perfusion of our model. In both conditions, the cells were cultivated inside the scaffolds for 21 days. Indeed, the formation of tubular structures was confirmed in both cases (21 days static culture and 21 days of dynamic culture at high flow rate). By utilizing 3D reconstruction of the images obtained using two-photon microscopy and second-harmonic generation imaging, we were able to demonstrate that the structures formed by the cells were hollow as neither columns of freeze-dried collagen nor fibroblasts could be found within the circumference of the tubular structures. However, comparing the static culture condition and dynamic cultivation at 425 µL/min, it was found that dynamic cultivation resulted in HUVECs forming a network that was independent from the scaffold structure and the fibroblasts in the samples (clearly visible by superimposing the red channel on the other channels investigated). Differences in the formation of vascular networks under static and dynamic conditions may be explained by shear-induced calpain activation, which leads to $Ca^{2+}$ entry through Piezo1 channels resulting in endothelial cell organization and alignment via actin cytoskeletal and focal adhesion proteins.[45]

Together, the results presented here support the contention that freeze-dried scaffolds are suitable materials for complex in vitro tissue models and offer benefits compared to collagen hydrogels: Although it has been shown that endothelial cells can self-assemble into network structures within hydrogels, they cannot be perfused without using technically difficult procedures such as templating[46,47] or sacrificial bioprinting.[48] In this investigation, we demonstrated the self-assembly of endothelial cells into hollow vessel-like structures without using any pre-patterning methods or a bioprinter, but simply by using a freeze-dried collagen type I scaffold. Beyond a high biocompatibility, the use of such scaffolds potentially allows the perfusion of the in vitro tissue model with suitable growth factors as previously investigated by Kolesky et al.[48] and enables further systematic studies on the effect of shear stress on self-assembly of endothelial cells into a vascular network. We suggest the use of other supporting cells such as primary fibroblasts, mesenchymal stem cells (MSCs) and pericytes to further investigate the interaction of these cells with HUVECs

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under the effect of perfusion. Moreover, studying the changes in concentration of biomarkers for angiogenesis such as Vascular Endothelial Growth Factor, angiopoietins and platelet-derived growth factor-BB could be interesting for further evaluation.

## 5 | CONCLUSIONS

It was shown that using a perfusable freeze-dried collagen scaffold enables the self-assembly of HUVECs into vessel-like structures for mesoscale in vitro tissue models. We confirmed that these vessel-like structures were hollow and demonstrated their formation in both static and dynamic culture conditions thus leading us to conclude that perfusion is not a necessary factor for prevascularization if hydrogel-permeated freeze-dried collagen scaffolds are used as an ECM. This simplifies future experimental procedures, as in vitro prevascularization can also be achieved without technically complex methods such as bioprinting or templating.

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### CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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