**Marquette University**

**e-Publications@Marquette**

***Biomedical Engineering Faculty Research and Publications/College of Engineering***

***This paper is NOT THE PUBLISHED VERSION*.**

Access the published version via the link in the citation below.

*Journal of Biomedical Materials Research. Part B : Applied Biomaterials*, Vol. 111, No. 3 (March 2023): 633-645. [DOI](https://doi.org/10.1002/jbm.b.35182). This article is © Wiley and permission has been granted for this version to appear in [e-Publications@Marquette](http://epublications.marquette.edu/). Wiley does not grant permission for this article to be further copied/distributed or hosted elsewhere without express permission from Wiley.

Development Of Fibroblast/Endothelial Cell-Seeded Collagen Scaffolds For In Vitro Prevascularization

Daniela S. Masson-Meyers

Marquette University School of Dentistry, Milwaukee, Wisconsin

Fahimeh Tabatabaei

Marquette University School of Dentistry, Milwaukee, Wisconsin

Lane Steinhaus

Marquette University School of Dentistry, Milwaukee, Wisconsin

Jeffrey M. Toth

Marquette University School of Dentistry, Milwaukee, Wisconsin

Lobat Tayebi

Marquette University School of Dentistry, Milwaukee, Wisconsin

# Abstract

The development of vascularized scaffolds remains one of the major challenges in tissue engineering, and co-culturing with endothelial cells is known as one of the possible approaches for this purpose. In this approach, optimization of cell culture conditions, scaffolds, and fabrication techniques is needed to develop tissue equivalents that will enable in vitro formation of a capillary network. Prevascularized equivalents will be more physiologically comparable to the native tissues and potentially prevent insufficient vascularization after implantation. This study aimed to culture human umbilical vein endothelial cells (HUVECs), alone or in co-culture with fibroblasts, on collagen scaffolds prepared by simple fabrication approaches for in vitro prevascularization. Different concentrations and ratios of HUVECs and fibroblasts seeded on collagen gel and sponge scaffolds under several culture conditions were examined. Cell viability, scaffolds morphology, and structure were analyzed. Collagen gel scaffolds showed good cell proliferation and viability, with higher proliferation rates for cells cultured in a 2:1 (fibroblasts: HUVECs) ratio and kept in endothelial cell growth medium. However, these matrices were unable to support endothelial cell sprouting. Collagen sponges were highly porous and showed good cell viability. However, they became fragile over time in culture, and they still lack signs of vascularization. Collagen scaffolds were a good platform for cell growth and viability. However, under the experimental conditions of this study, the HUVEC/fibroblast-seeded scaffolds were not suitable platforms to generate in vitro prevascularized equivalents. Our findings will be a valuable starting point to optimize culture microenvironments and scaffolds during fabrication of prevascularized scaffolds.

# 1 INTRODUCTION

The main goal of tissue engineering is to replace injured or lost tissues with equivalents that will mimic their structure and function while enhancing tissue repair.1, 2 An essential part of the survival and final success of the reconstructed tissues, particularly during the repair of critical-sized tissues defects, is the development of a vascular network in the tissue equivalents. However, the development of vascularized scaffolds remains one of the greatest challenges in regenerative medicine.

A successful tissue equivalent implantation will depend on the appropriate integration of a vascular network capable of providing adequate diffusion of oxygen and nutrients.3, 4 Cells seeded inside a scaffold and the host cells surrounding the graft should be in close proximity to a capillary (approximately 100–200 μm, depending on the tissue) to ensure long-term survival and function.2, 5-8 When the implant is small, the hosts' pre-existing vasculature can supply enough oxygen and nutrients. However, in cases of large defects, this diffusion might not be sufficient to reach the center of the scaffold, potentially leading to tissue necrosis and implant failure.1

One of the alternatives to overcome this issue is to fabricate vascularized scaffolds. Several strategies have been developed to create a functional vascular network and promote vascularization in tissue engineering applications, including angiogenic growth factor delivery, optimization of scaffold properties, in vivo and in vitro prevascularization.1, 4, 7-11 In vitro prevascularization (i.e., pre-formation of a capillary network in the tissue equivalents before in vivo implantation) can be achieved by different approaches such as cell sheet technology, development of spheroids and scaffold cell seeding. The cell seeding approach involves the seeding of endothelial cells usually with supporting cells on scaffolds, and was the approach used for this current study. Once the construct is vascularized in vitro, its implantation in vivo will likely provide a beneficial environment for prompt integration with the host tissue and further vascularization.1, 4, 10, 12

The design and fabrication of appropriate biomaterials that enable vascularization still face multiple challenges, and the studies on scaffold prevascularization are very heterogeneous, making it difficult to adapt a protocol to a specific tissue construct.1, 13 Among the challenges associated with developing these scaffolds, the choices of appropriate scaffold materials, cells, and culture conditions are critical for cell-based vascularization strategies.

The design of a scaffold with optimal characteristics is one of the most critical elements for a successful tissue engineering application.13 One of the most significant guides in the scaffolds' design is that biomaterials should resemble the natural extracellular matrix (ECM)—the extracellular component of natural tissues composed mainly of collagen, elastin, integrins, and laminins, that provide spatial and mechanical signals to cells and structural and biochemical support to tissues.13-15 By mimicking the natural ECM of an injured tissue, scaffolds are expected to support cellular proliferation and differentiation to repair it.6, 14 Scaffolds should have adequate porous architecture and interconnectivity to allow cell infiltration, proliferation, vascularization, cell–cell communication, and cell culture medium perfusion. Moreover, they should be biocompatible, biodegradable, non-toxic, non-allergenic, and bio-absorbable with adequate mechanical properties.7, 16

Different types of hydrogels have been used as 3D supporting matrices to mimic the native ECM physiological microenvironment due to their unique compositional and structural similarities to the natural ECM and tunable physicochemical properties.13, 16-18 Hydrogels can be derived from natural or synthetic sources. Natural polymers including collagen, gelatin, chitosan, hyaluronic acid, chondroitin sulfate, alginate, and fibrin are the most used biomaterials to fabricate hydrogels in soft tissue engineering due to their biocompatibility, biodegradability, and low cytotoxicity. However, synthetic biomaterials such as poly (ethylene) glycol (PEG) and polylactic acid (PLA), can be combined to improve the stability and mechanical properties of the natural polymeric hydrogel.17, 19-25

Collagen is considered one of the most essential biomaterials in connective tissue repair because of its hydrophilicity, excellent biocompatibility, low immunogenicity, flexibility, and chemotaxis. Due to its biomimetic and structural composition in the extracellular matrix, collagen-based scaffolds can support cell responses, such as cellular adhesion, migration, and proliferation.26-28 Although collagen is largely responsible for the tensile properties of native connective tissues, collagen hydrogels have relatively poor mechanical properties. Thus, many studies combine collagen with other materials using cross-linking agents or sophisticated fabrication techniques.6, 19, 29, 30

To the best of our knowledge, there has not been a study that has reported the use of a simple cell-seeded hydrogel scaffold fabricated by a classic/simple casting technique, aiming at in vitro prevascularization. It is crucial to understand scaffolds' properties and how they influence the behavior of endothelial cells (ECs) cultured alone or in combination with supporting cells (co-cultures), and if they would support capillary network formation. Therefore, simply designed scaffolds can provide insights that could not be possible if they were modified for instance, by adding growth factors or other materials that would alter their composition and/or structure.

Once the appropriate biomaterial for a specific study has been determined, the cells and culture conditions should be established. Physiologically, angiogenesis involves a complex signaling pathway and several cells are involved, as they provide ECs with pro-angiogenic factors required for their migration and capillary formation.3, 10 Therefore, for in vitro vascularization strategies, ECs are usually co-cultured with supporting pro-angiogenic cells (e.g., fibroblasts, pericytes, mesenchymal stem cells, vascular smooth muscle cells).3, 6, 10-12, 16, 19, 22, 31, 32

Cell types, co-cultures, source, seeding technique, number and location within the scaffolds are also essential to be determined. A variety of EC subtypes are currently in use in prevascularization studies, including HUVECs, human microvascular endothelial cells (HDMECs), endothelial progenitor cells (EPCs), and endothelial colony-forming cells (ECFCs).22, 23, 29, 30, 33, 34 HUVECs are the most common choice due to the relatively easy accessibility, low immunogenicity, and ease of culture.16 The co-culture of ECs with fibroblasts and/or other supporting cells, embedded in an appropriate biomaterial is a promising strategy of in vitro prevascularization to enhance angiogenesis, and provide adequate nutrient and oxygen supply after transplantation.6

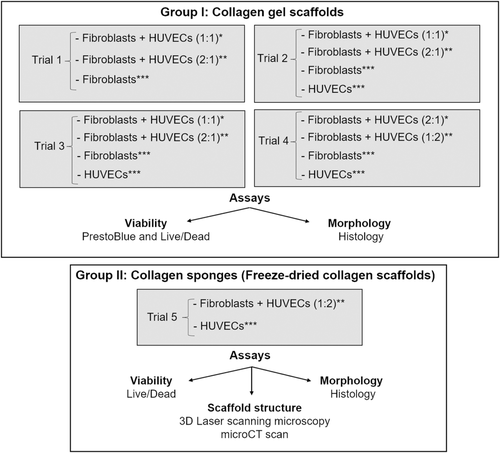
Notably, along with the selection of biomaterial, ECs, and supporting cells, culture conditions should be carefully determined to ensure that cells have appropriate nutrients to grow and that the cell culture medium does not affect the scaffold's properties or influence the accuracy of the assays performed during their in vitro evaluation. However, protocols differ among studies and there are no specific culture conditions, especially when using co-culture systems.12

Thus, this study aimed to determine if simply fabricated collagen scaffolds would support the culture or co-culture of HUVECs and/or fibroblasts and be a suitable scaffold to promote in vitro capillary formation. We sought to develop scaffolds by simple preparation approaches, such as hydrogel casting and cell seeding, without the addition of growth factors or combination of materials, functionalization, or other sophisticated fabrication strategies. The purpose of this was to assess the response of HUVECs and/or fibroblasts to the scaffold materials alone. Also, a simple structured hydrogel could be a more reproducible system and its fabrication could be possible, for example, for laboratories with limited resources. This study provides insight into some of the challenges of generating prevascularized soft tissue constructs. These challenges are often briefly mentioned in the literature, but not reported in detail.

# 2 MATERIALS AND METHODS

## 2.1 Study overview

Experimental trials performed in this study are grouped according to the scaffold structure. Figure **1** outlines the two main groups: I. Collagen gel and II. Collagen sponges, and the different experimental conditions that were tested.

[](https://onlinelibrary.wiley.com/cms/asset/574466c9-ade9-482e-8b46-89df3295d7d5/jbmb35182-fig-0001-m.jpg)

**FIGURE 1** Study design. HUVECs: human umbilical vein endothelial cells. \*5 × 105 cells/scaffold for each cell line; \*\*5 × 105 and 2.5 × 105 cells/scaffold for each cell line; \*\*\*5 × 105 cells/scaffold

## 2.2 Cell culture

Cell lines used in the study included human gingival fibroblasts and human umbilical vein endothelial cells. Fibroblasts were obtained from liquid nitrogen storage at Marquette University School of Dentistry. These cells were previously isolated from small biopsies obtained from healthy patients undergoing oral surgery after informed consent and approval by the Institutional Review Board at Marquette University, as described by Tabatabaei et al.35 HUVECs obtained from pooled donors were purchased from Lonza (CC2519).

Fibroblasts were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Corning®, 15-017-CV) supplemented with 10% fetal bovine serum (FBS), 2 mM l-Glutamine and 1% antibiotic-antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). HUVECs were cultured in endothelial cell growth medium-2 BulletKit™ (EGM™-2, CC-3162, Lonza), which consists of endothelial cell growth basal medium-2 (EBM™-2, CC-3156, Lonza) supplemented with EGM™-2 SingleQuots™ Kit (CC-4176, Lonza). The final complete medium contained 2% FBS, hydrocortisone, human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-b), recombinant human insulin-like growth factor (R3-IGF-1), ascorbic acid, heparin, and gentamicin sulfate/amphotericin B (GA-1000).

Both cell lines were cultured in 75 cm2 cell culture flasks and maintained in a controlled humidified cell culture incubator (37°C, 5% CO2). The culture medium was changed every 2 days. When cells became confluent, the medium was removed and the cell layer trypsinized with 0.25% trypsin in buffered ethylenediaminetetraacetic (EDTA). Cells were mixed with Trypan blue (1:1 ratio), applied into the counting chamber of a disposable hemocytometer (C-Chip DHC-N01-5 Neubauer improved [iNCYTO]), and counted under the microscope. Suspensions with different cell concentrations were prepared for seeding. Passages 3–6 were used for the experiments.

## 2.3 Scaffolds preparation and cell seeding

### 2.3.1 Collagen gel scaffolds

Type I bovine collagen (6 mg/ml; Nutragen, Advanced Biomatrix) was mixed on ice with 10× DMEM (13.8 mg/ml) with or without phenol red, 10× reconstitution buffer (2.25 mg/ml [22 mg/ml sodium bicarbonate and 20 mM HEPES in 0.062 N NaOH]), FBS (8.5% v/v) and l-Glutamine (2 mM). After mixing the solution carefully to avoid bubbles, the pH was adjusted to 7.4 with sterile sodium hydroxide. Cells at different concentrations and ratios were added to the solution and gently mixed. Cell concentration was 5 × 105 cells/scaffold for each cell line (1:1 ratio); 5 × 105 and 2.5 × 105 cells/scaffold (2:1 ratio) and 5 × 105 cells/scaffold for one cell line. Four different trials were tested for this group of scaffolds:

1. Fibroblasts + HUVECs at 1:1 and 2:1 ratios and fibroblasts only. Cultures were maintained in DMEM with phenol red.
2. Fibroblasts + HUVECs at 1:1 and 2:1 ratios, fibroblasts only and HUVECs only. Cultures were maintained in phenol red-free DMEM.
3. Fibroblasts + HUVECs at 1:1 and 2:1 ratios, fibroblasts only and HUVECs only. Cultures were maintained in DMEM:EGM-2 (1:1).
4. Fibroblasts + HUVECs at 2:1 and 1:2 ratios, fibroblasts only and HUVECs only. Cultures were maintained in EGM-2.

Once the solution was homogenous, it was distributed into tissue culture inserts (0.4 μm pore size, 24 mm diameter, VWR) in 6-well plates, using a volume of 2.5 ml/insert. Plates were transferred to a cell culture incubator (37°C, 5% CO2) for 2 h to allow polymerization of collagen. Once the constructs were solidified, 3 ml of warmed cell culture medium was added per well (1.5 ml on top of the gel/inside the insert and 1.5 ml outside/under it) (i.e., bottom of the well). Plates were maintained in the cell culture incubator and the medium was changed every 2 days.35-37

### 2.3.2 Collagen sponges

The first step during the preparation of collagen sponges was to make a collagen solution using the same formulation and procedure described above, but no cells were added (acellular collagen). Once the scaffolds were solidified, after 2 h at 37°C, the plates were transferred to −20°C for 24 h. Next, samples were freeze-dried at −52°C, 1 Pa for 48 h (Labconco 2.5-liter benchtop freeze dry system). Before seeding the cells, both sides of the scaffolds were sterilized under UV light for 1 h (30 min/side).

After sterilization and before cell seeding, the scaffolds were rehydrated by adding the cell culture medium and 6-well plates were incubated at 37°C for 30 min. Meanwhile, cell suspensions were prepared: fibroblasts + HUVECs (1:2 ratio) and HUVECs only. Cell concentration was 2.5 × 105 and 5 × 105 cells/scaffold (1:2 ratio) and 5 × 105 cells/scaffold for HUVECs only. Once the cell suspensions were ready, the culture medium was removed from the scaffolds, cells were seeded (500 μl cell suspension/scaffold), and plates were incubated (37°C, 5% CO2) for 30 min to allow cell attachment to the scaffolds. Then, 3 ml of warmed EGM-2 was added per well (1.5 ml inside and 1.5 ml outside the insert). Plates were maintained in the cell culture incubator, and the medium was changed every 2 days until constructs were used for an assay.

## 2.4 Assays

### 2.4.1 Cell viability

Viability of fibroblasts and HUVECs seeded in the scaffolds was assessed by PrestoBlue and live/dead assays.

### PrestoBlue assay

At different time points, the culture medium was removed from the wells and the scaffolds were rinsed once with sterile phosphate-buffered saline (PBS). Then, a volume of 1 ml of a 1:10 solution [PrestoBlue reagent (Invitrogen, A13626):phenol red-free DMEM (HyClone, SH3028401)] was added to each well, and the plate was incubated at 37°C for 3 h. Aliquots of 200 μl taken in triplicate from each well/scaffold were transferred to a 96-well plate. The fluorescence intensity was measured at 540 nm excitation, 590 nm emission using a microplate reader (BioTek Synergy HTX plate reader).38 Once the measurements were completed, scaffolds were rinsed once with sterile PBS, fresh culture medium was added, and plates were returned to the incubator until the next time point/measurements. The results are presented as mean ± SD of fluorescence intensity measurements (*n* = 3).

### Live/dead viability assay

Collagen scaffolds (gel and sponges) were transferred from the inserts/wells to another 6-well plate without inserts, and carefully rinsed twice with sterile PBS (2–3 ml PBS/well) before they were manually sectioned in smaller fragments with a scalpel. Sections were transferred to a 24-well plate and washed twice with sterile PBS (2–3 ml PBS/well) for 5 min. After aspiration of PBS, a volume of 2 ml working solution containing 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM following manufacturer's instructions (Live/Dead viability kit [Invitrogen, Cat#L3224]) was added. Plates were covered with aluminum foil and incubated at 37°C/5% CO2 for 30 min. After removing solution and washing the sections twice with sterile PBS for 5 min, sections were placed onto microscope glass slides and imaged using a fluorescence microscope (EVOS® FL Auto, lifetechnologies). The results are shown as representative fluorescent photomicrographs.

### 2.4.2 Histological analysis

After 7 days in culture, samples were rinsed twice in PBS and fixed in 10% neutral buffered formalin for 2, 24 or 48 h, at 4°C. Samples were transferred to embedding cassettes between two pieces of biopsy sponges, and dehydrated overnight using a bench top tissue processor (KD-TS1A, Keede) and embedded in Paraplast tissue embedding medium (McCormick Scientific) using a tissue embedding center (KD-BM II, Keede). Samples were stored at −20°C. After deparaffinization and rehydration, 10 μm sections collected on positively charged microscope slides, were stained with Hematoxylin and Eosin (H&E) and examined using an inverted microscope (EVOS® FL Auto, lifetechnologies). The results are shown as representative photomicrographs.

### 2.4.3 Morphological and structural characterization of collagen sponge scaffolds

To analyze the scaffolds' structure and pore formation after the freeze-drying process, collagen sponges were subjected to 3D laser scanning microscopy. Dry samples without cells were sectioned in smaller fragments with a scalpel, and images were acquired at a 5× magnification (Olympus LEXT 3D measuring laser microscope OLS 4000, Japan).

Additionally, the pore structures of the collagen sponges were analyzed by a high-resolution micro-computed tomography (micro-CT) scanner (Skyscan Micro Photonics, Belgium). Samples were sectioned with a scalpel and positioned horizontally on a brass holder in the center of the specimen stage. Cross-sectional images were reconstructed and the numerical data was calculated using SkyScan 1172 software.37

## 2.5 Statistical analysis

Results from the PrestoBlue viability assay were expressed as fluorescence intensity and presented as mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test (GraphPad Prism 8.00 software, GraphPad Software Inc., USA). The level of statistical difference was set at *p* < 0.05.

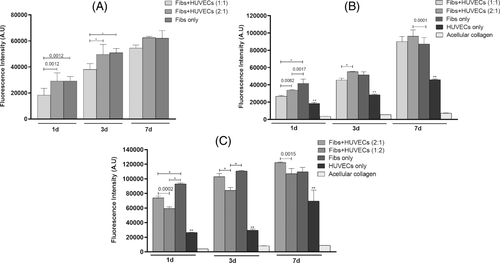
# 3 RESULTS

## 3.1 Collagen gel scaffolds (Group I)

Cell viability in the collagen gel scaffolds was analyzed by PrestoBlue and live/dead assays, and morphology was assessed by histology using H&E staining.

### 3.1.1 PrestoBlue

The first set of collagen gel scaffolds seeded with fibroblasts and HUVECs were kept in culture using DMEM medium (trial 1). Three groups were evaluated: Fibroblasts + HUVECs (1:1), Fibroblasts + HUVECs (2:1) and fibroblasts only. The scaffolds containing fibroblasts only and a co-culture of fibroblasts:HUVECs (2:1) showed a higher cell proliferation rate compared to the 1:1 ratio, more so after 1 day (*p* = 0.0012, *p* = 0.0012, respectively) and 3 days (*p* < 0.0001, *p* < 0.0001, respectively) of culture. However, the difference was no longer statistically significant after 7 days of culture (Figure **2A**). Additionally, this assay did not distinguish between cell lines in the co-culture groups. Thus, for the subsequent trials, a HUVECs-only group was included. Figure **2B** represents the results from trial 2 and shows a similar trend, with the 2:1 ratio (Fibroblasts:HUVECs) showing a higher proliferation rate than the 1:1 ratio, more so after 1 day (*p* = 0.0017) and 3 days (*p* < 0.0001) of culture. Also, the group containing HUVECs only showed a lower proliferation rate than other groups in all time points (2, 4, and 7 days), *p* < 0.0001. This was expected because HUVECs grow slower than fibroblasts. Moreover, scaffolds in this trial were kept in a DMEM medium, which could have influenced HUVECs growth. For the following trial (trial 3), a combination of DMEM and EGM-2 (50% each medium) was used, and the results were similar (data not shown). Then, for next trial (trial 4), EGM-2 medium was used for all scaffolds (Figure **2C**). In this trial, the ratio of 1:1 (Fibroblasts:HUVECs) was excluded, and a ratio of 1:2 (Fibroblasts:HUVECs) was included. HUVECs showed a better proliferation rate after 7 days in culture when cultured alone in the scaffolds compared to trials where the seeded scaffolds were maintained in DMEM or DMEM + EGM-2. The inclusion of a higher ratio of HUVECs in co-culture with fibroblasts (1:2, Fibroblasts:HUVECs) still showed a lower proliferation rate after 1 and 3 days in culture compared to the 2:1 ratio (Fibroblasts:HUVECs) (*p* = 0.0002, *p* < 0.0001) or fibroblasts only (*p* < 0.0001, *p* < 0.0001). After 7 days in culture, cell proliferation of the 1:2 ratio (Fibroblasts:HUVECs) was not statistically significant from fibroblasts-only scaffolds. From this assay, EGM-2 as the cell growth medium and cell ratio of 2:1 (Fibroblasts:HUVECs) could be a good starting point to optimize scaffolds/co-culture conditions to yield in vitro prevascularization.

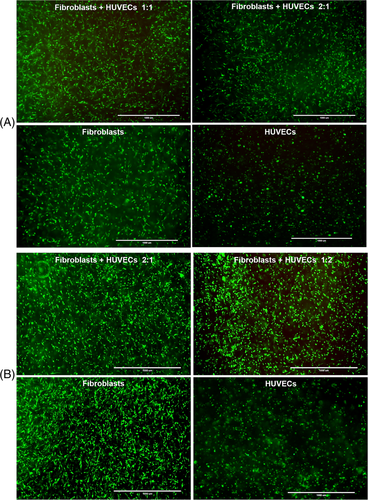
[](https://onlinelibrary.wiley.com/cms/asset/3c4a6d58-c01e-4c8d-9bf5-ee7bf320fe20/jbmb35182-fig-0002-m.jpg)

**FIGURE 2** Cell viability/proliferation rate of seeded collagen gels under different culture conditions analyzed by PrestoBlue assay at 1, 3 and 7 days of culture. (A) Co-culture of fibroblasts and HUVECs at 1:1 and 2:1 ratios, and monoculture of fibroblasts; all maintained in DMEM culture medium. (B) Co-culture of fibroblasts and HUVECs at 1:1 and 2:1 ratios, monocultures of fibroblasts and HUVECs, and a group containing only the collagen formulation (acellular collagen); all maintained in phenol red-free DMEM culture medium. (C) Co-culture of fibroblasts and HUVECs at 2:1 and 1:2 ratios, monocultures of fibroblasts and HUVECs, and acellular collagen; all maintained in EGM-2 culture medium. Results are presented as mean ± SD fluorescence intensity (*n* = 3). \*Represents *p* < 0.0001; \*\*Represents the statistical difference between HUVEC-seeded collagen gels compared to the other groups at each time point (*p* < 0.0001)

### 3.1.2 Live/dead viability assay of collagen gel scaffolds

For the first trial, the culture medium and the medium used to make the collagen gel scaffolds was DMEM with phenol red. This medium caused background fluorescence when scaffolds were visualized under the RFP filter (data not shown). At first, the DMEM used to make a 10× solution added in the collagen formulation was replaced with a phenol red-free medium, but the red background was still present. Next, the DMEM growth medium was also replaced with a phenol red-free version. With this approach, it was possible to analyze the scaffolds and visualize red-stained cells, that is, dead cells stained by EthD-1, without the red background fluorescence. It is noteworthy to mention that different concentrations of EthD-1 were assessed to prepare the working solution. However, even at lower concentrations than what is recommended by the manufacturer, the red background was still present.

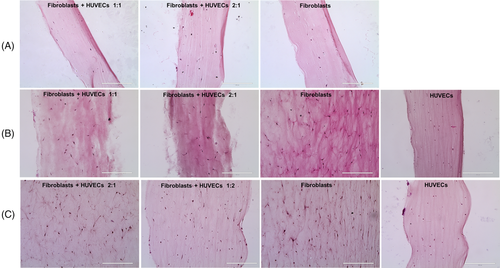
Once the protocol was optimized, the viability of fibroblasts and HUVECs in the scaffolds was assessed. Results of this assay were consistent with the proliferation assay and showed live cells in all collagen gel scaffolds, confirming the excellent biocompatibility of collagen. Some culture conditions were better than others. For example, more cells were observed in the scaffolds cultured with the EGM-2 medium, and HUVECs co-cultured with fibroblasts, especially at a 1:2 ratio, showed higher viability than HUVECs cultured alone in the scaffolds. Figure **3A** represents the trial where the scaffolds were maintained in DMEM, and Figure **3B** represents the trial where the scaffolds were maintained in EGM-2 medium. Live/dead cell analysis also revealed a uniform distribution of cells throughout the scaffolds.

[](https://onlinelibrary.wiley.com/cms/asset/0cb6d2d4-33a3-48b1-8521-a792226808e2/jbmb35182-fig-0003-m.jpg)

**FIGURE 3** Representative fluorescent photomicrographs of live/dead staining of seeded collagen gels after 7 days in culture. (A) Collagen gels cultured in DMEM. (B) Collagen gels cultured in EGM-2. Live cells were stained with calcein AM (represented by green fluorescence) and captured using a GFP filter. Dead cells were stained with ethidium homodimer-1 (represented by red fluorescence) and captured using an RFP filter. Scale bar: 1000 μm. Magnification 4×

### 3.1.3 Histological analysis of collagen gel scaffolds

The morphology of collagen gel scaffolds collected after 7 days in culture and fixed in 10% neutral buffered formalin for 2, 24, or 48 h, and stained by H&E is represented in Figure **4A,B,C**, respectively. Results show that by increasing samples fixation time, more cells were observed in the scaffolds. Consistent with PrestoBlue and live/dead assays, co-cultures of fibroblasts + HUVECs (2:1) in the EGM-2 medium showed more cells than other groups (Figure **4C**). However, the number of cells was lower than the number of cells visualized by live/dead staining, indicating a possible cell lost during the histological processing. Further, none of the samples showed evidence of capillary formation.

[](https://onlinelibrary.wiley.com/cms/asset/1ac78057-2779-4473-8498-7de7cdc790cb/jbmb35182-fig-0004-m.jpg)

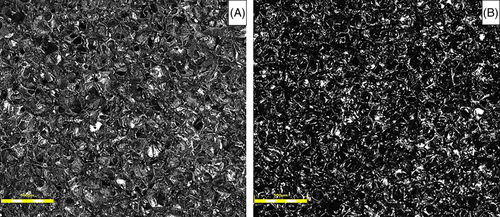
**FIGURE 4** Representative photomicrographs of Hematoxylin and Eosin-stained seeded collagen gels after 7 days in culture, fixed in neutral buffered formalin for different times. (A) 2 h; (B) 24 h; (C) 48 h. Scale bar: 200 μm. Magnification 20×

## 3.2 Collagen sponge scaffolds (Group II)

Aiming at introducing pores in the previous collagen gel scaffolds to possibly obtain a better platform to support HUVECs to form a capillary network, their structure was modified by lyophilization. Prior to analyses of cell viability (live/dead) and morphology (histology), the structure of acellular sponges was analyzed by laser microscopy and micro-CT scan.

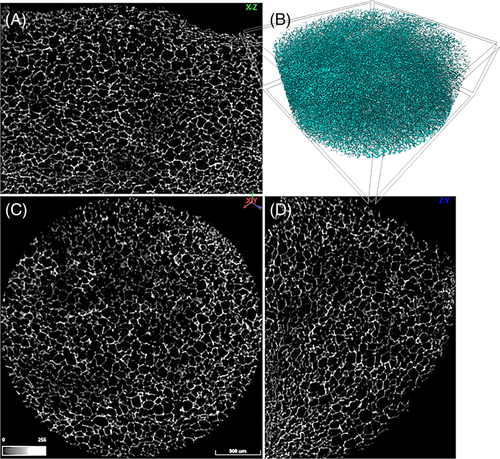
### 3.2.1 Structure of collagen sponges: Laser and micro-CT scan

An initial analysis of the scaffolds structure was carried out by laser microscopy. Images of the top and bottom were acquired. The scaffolds were highly porous, and the pores were well distributed. Images from the top of the sponges showed the absence of a smooth layer, indicating that the lyophilization/freeze-drying process was adequate (Figure **5**).

[](https://onlinelibrary.wiley.com/cms/asset/2e8d418a-3a66-45f9-af6a-2f374c8693ef/jbmb35182-fig-0005-m.jpg)

**FIGURE 5** Laser microscope representative photomicrographs of freeze-dried collagen scaffolds from (A) Top and (B) Bottom. Scale bar: 500 μm. Magnification 5×

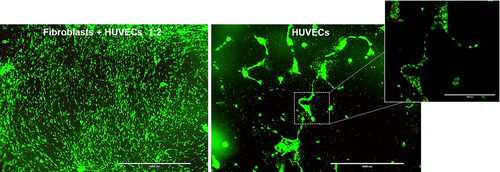
Results from the micro-CT analysis corroborated laser microscopy results, showing high porosity with pores relatively homogenous throughout the scaffold. The μ-CT and 3D reconstruction image analysis of the scaffolds demonstrated pore size distribution in 2D (Figure **6A,C,D**) and the 3D interconnectivity of the porous structure (Figure **6B**). The pores average diameter was 435 μm, and the porosity was 85%.

[](https://onlinelibrary.wiley.com/cms/asset/cfc2cd0f-a2d7-422d-a6df-752cf4ca3068/jbmb35182-fig-0006-m.jpg)

**FIGURE 6** Representative micro-CT images of different cross-sections of freeze-dried collagen scaffolds. (A) 2D structure of scaffold *X*–*Z*. (B) 3D structure of scaffold. (C) 2D structure of scaffold *X*–*Y*; (D) 2D structure of scaffold *Z*–*Y*. Scale bar: 900 μm

### 3.2.2 Live/dead viability assay of collagen sponges

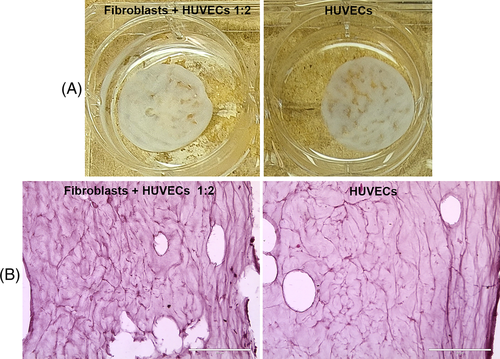
Similar to the results from the collagen gels, the live/dead assay revealed high viability of a co-culture of fibroblasts and HUVECs (1:2 ratio) and HUVECs only in the collagen sponges. Interestingly, the staining of the scaffolds seeded with HUVECs only showed that they were starting to attach around the pores instead of being dispersed throughout the scaffold, as observed in the collagen gels scaffolds (Figure **7**).

[](https://onlinelibrary.wiley.com/cms/asset/44b6fc71-2cb1-4827-adc5-2755edd85f6c/jbmb35182-fig-0007-m.jpg)

**FIGURE 7** Representative fluorescent photomicrographs of live/dead staining of fibroblasts and/or HUVECs seeded on collagen sponges after 7 days in culture. Live cells were stained with calcein AM (represented by green fluorescence) and captured using a GFP filter. Dead cells were stained with ethidium homodimer-1 (represented by red fluorescence) and captured using an RFP filter. Scale bars: 1000 μm and 400 μm, magnification 4× and 10× for large and small images, respectively

### 3.2.3 Macroscopic and histological analyses of collagen sponges

Scaffolds became very fragile over time during incubation in cell culture medium, as shown in Figure **8A**. The porous structure was observed after H&E staining. However, cells were not present in the sections (Figure **8B**). Due to the scaffolds being so thin, steps such as washes before fixation, or the dehydration process of unfixed cells might have washed them off.

[](https://onlinelibrary.wiley.com/cms/asset/46f82f95-7159-4221-b5b0-e7227f43ab20/jbmb35182-fig-0008-m.jpg)

**FIGURE 8** (A) Representative images of fragile collagen sponge scaffolds after 7 days in culture. (B) Representative photomicrographs of Hematoxylin and Eosin-stained seeded collagen sponges after 7 days in culture. Scale bar: 200 μm. Magnification 20×

# 4 DISCUSSION

The purpose of this study was to fabricate a simple scaffold that would support the growth of HUVECs or their co-culture with fibroblasts and promote in vitro prevascularization. In the experimental conditions evaluated in our study, collagen scaffolds were a good platform for HUVECs and fibroblasts proliferation and viability. However, they were not able to support endothelial cell sprouting. Various elements such as scaffold structure and culture settings, including variations in cell density and ratio, and cell culture growth medium were tested.

The challenges associated with in vitro prevascularization are a common subject described in several publications. However, to the best of our knowledge, most of these challenges are not reported, as they can be perceived as experimental failures. This study presented our attempts and difficulties in designing in vitro prevascularized scaffolds. We hope that our findings will potentially expedite the achievement of positive outcomes by decreasing the duplication of efforts by other investigators. Moreover, assays were optimized, which will assist other researchers with their investigation, for instance, of scaffolds' biocompatibility, morphology, and structure.

In the first few trials, we developed collagen gels seeded with different concentrations and ratios of fibroblasts and/or HUVECs. Proliferation assay showed that cells were viable for all conditions tested, with higher proliferation when cells were cultured in a 2:1 (fibroblasts:HUVECs) ratio, and scaffolds were kept in culture with the EGM-2 medium. The next step was to assess cell viability and their distribution in the scaffolds by fluorescent staining using the live/dead assay. The phenol red present in the DMEM (either in the collagen formulation or as the cell growth medium) prevented the collagen gels to be visualized after live/dead staining. We replaced DMEM with phenol red-free DMEM from the formulation only and still had a red background, which was removed when we replaced both sources of DMEM (from formulation and cell growth medium). We then were able to check cell viability inside the scaffolds. The next step was to check if HUVECs formed capillary-like structures in the scaffolds and samples were processed for histological analysis (H&E staining). The first set of samples were fixed in formalin for 2 h and after staining, only a few cells were observed. As the fixation time increased to 24 and 48 h for the next samples, it was possible to see more cells in the H&E-stained sections. However, it was not as many cells as expected and there was no evidence of capillary formation. Seeded scaffolds were rinsed with PBS before being fixed with formalin. It is possible that some cells were washed off during this process or if they were not properly fixed, the dehydration process could have removed them from the scaffolds.

Different cell concentrations and ratios were assessed on collagen scaffolds, but no vessel formation was achieved. Several factors could have influenced the outcomes of these experiments. For instance, collagen scaffolds were cast in 6-well culture plates, and it is possible that their size was too large for the cell densities tested. As a further investigation, they could be cast in 24-well plates instead for comparison, as used in similar in vitro prevascularization studies.6, 20, 29, 33 Cell density could have been another factor that can be improved in future studies. A higher cell density (e.g., 3, 5 or 10 × 106 cells/ml) could facilitate cell–cell contact and possibly vascular network formation.20, 39

The final collagen concentration in our scaffolds was 4 mg/ml. This might have been too high, possibly making them stiff, which could have impaired HUVECs migration and eventually capillary network formation. However, this assumption is hypothetical since we did not characterize scaffolds' stiffness. A study by Byfield et al.,40 considered a 3 mg/ml (502 Pa) type I collagen gel as a stiff gel, while gels composed of 1.0–1.5 mg/ml type I collagen (124–202 Pa) were compliant. Endothelial cells formed microvascular-like networks in both gels; however, the cells were stiffer in the stiff gel, showing that extracellular substrate stiffness regulates endothelial cell stiffness. Further, HUVECs migration might have also been affected by the scaffold's architecture. To address some of these concerns, we fabricated the same scaffold, but modified its structure by freeze-drying.

Freeze-drying has been extensively used to produce porous hydrogel matrices. For example, this approach to lyophilize collagen-based scaffolds is commonly used because it can maintain the collagen's native structure and biological properties. Moreover, introducing pores in the scaffolds can improve cell migration.13, 26

Appropriate porous structures are required to improve the capability of ECs to promote angiogenesis.5 Proper porosity provides a physical surface for cell migration, proliferation, cell–cell contact, and cell-matrix interactions, facilitating the formation of a vascular network in vitro and in vivo. Furthermore, interconnected pores provide improved nutrient and oxygen supply to the center of the scaffold, thus reducing the chances of developing a necrotic center.4 However, excessive porosity might result in reduced mechanical strength.

The collagen sponge scaffolds were highly porous and the pores were homogeneously dispersed throughout the scaffolds, indicating promising results from a structural standpoint. Similar to the cell viability in the collagen gel scaffolds, the sponges showed excellent biocompatibility after 7 days in culture. Moreover, HUVECs seemed to be attached around the pores/pores interconnection, which has been reported to be the site where ECs migrate to the inner part scaffolds to induce vascularization.5 However, the introduction of porosity made these scaffolds very thin and fragile over time in culture and again, histological findings did not show any evidence of capillary formation. Due to the scaffolds being so thin, it is possible that the cells could have been disturbed/removed during washes before formalin fixation or during the dehydration process. Another hypothesis is that the pore size might have been large (mean pore size was 435 μm), but pore sizes in in vitro prevascularization studies using similar collagen scaffolds vary, for instance, from 20–40 μm,30 80 μm29 to 325 μm.41 Cross-linking these scaffolds or the fabrication of composite/hybrid scaffolds could be the next steps to improve these collagen scaffolds as an attempt to provide a better platform for in vitro prevascularization. Additionally, a combination of freeze-drying with foaming that leads to 3D dual-porosity scaffolds, could be another approach to produce porous protein-based scaffolds with less shrinkage and deformation with better diffusion kinetics and has been shown to improve cell proliferation.42

Engineered hydrogels derived from natural proteins such as collagen are known to facilitate ECs migration during angiogenesis and they would be the preferred choice due to their potential to mimic the natural ECM. However, most of them have limited strength, especially if used alone.3 During the development of tissue equivalents, prevascularized or not, it is important to enhance the physical properties of the scaffolds and to facilitate cellular activities.16 Surface (chemical composition, topography), mechanical and morphological properties of the scaffold are critical cues for directing cellular behavior.14

Some of the approaches to improve mechanical properties and the structure of scaffolds include cross-linking (e.g., using N-[3-dimethylaminopropyl]-N′-ethylcarbodiimide and N-hydroxysuccinimide), use of composite collagen-based scaffolds (e.g., collagen-chitosan, collagen-glycosaminoglycan, collagen-glycosaminoglycan-chitosan, collagen-chondroitin sulfate, and collagen-agarose), and a combination of natural and synthetic polymers (e.g., gelatin-methacrylate [GelMA]).16, 21 Another approach that could optimize capillary formation and stabilization is the use of co-cultures of ECs with fibroblasts, pericytes, and smooth muscle cells.9, 19, 31, 43 In regards to scaffold cell seeding, one alternative strategy is the combination of HUVECs with fibrin gel before adding them to the scaffolds, which could support the co-culture of HUVECs and supporting cells, and enhance capillary-like structure formation.29, 32 Additionally, another suggested approach is sequential cell seeding technique instead of seeding cells simultaneously. For instance, seeding fibroblasts before seeding ECs could allow for ECM formation and growth factor release to enhance ECs growth.33 Lastly, another change in culture condition that might assist with capillary network formation could be the use of a bioreactor to simulate the physiological in vivo environment.21, 22, 44

Our goal in this study was to use straightforward protocols to fabricate a construct that would be a suitable environment to co-culture ECs and fibroblasts for in vitro prevascularization. However, in vitro vascularization is a complex process. Several scaffold production techniques, scaffold materials, different cell sources, and culture conditions have been described in the literature, and no consensus exists, making this process particularly challenging. It would be more easily reproducible if we could get a scaffold that is produced, for instance, only by simply casting collagen and cells, without the need for further modifications such as functionalization, composite scaffolds, sophisticated techniques (3D printing, microfluidics, electric spinning, layer-by-layer technology), or incorporation of pro-angiogenic growth factors (e.g., VEGF).

Our findings and available literature show that prevascularization strategies are intricate, fabrication of 3D prevascularized equivalents is still challenging, and protocols require further modifications. Depending on the application, structural complexity and technique improvements might still be required to warrant the in vitro capillary network formation.

# 5 CONCLUSION

Under the conditions examined in this study, collagen scaffolds supported the culture and co-culture of fibroblasts and HUVECs. HUVECs cultured or co-cultured with fibroblasts in collagen gel or sponge scaffolds did not result in capillary-like structure formation. However, in both structures, high cell proliferation and survival rates were obtained.

A myriad of factors make the development of in vitro prevascularized tissue-engineered constructs still a challenge in the field, and our findings and difficulties depicted a few of them. The outcomes of our study will be beneficial for future optimization of scaffolds and culture microenvironments. Further modifications in the collagen scaffold formulation, fabrication technique, and culture conditions are needed to find the most suitable conditions that will enable the formation of capillaries in vitro (prevascularization) before implantation and contribute to a better chance of integration and improved vascularization and tissue repair. Using innovative technologies—such as organ-on-a-chip, bioprinting, and micro-patterning design of scaffolds—could help to achieve these goals.

# ACKNOWLEDGMENTS

This work was supported by the National Institute of Dental & Craniofacial Research/National Institutes of Health (NIDCR/NIH), award numbers R15DE027533, 3R15DE027533-01A1 and 1R56 DE029191-01A1. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

# CONFLICT OF INTEREST

Author J.M.T. serves as scientific consultant to Medtronic, Ossifi Inc., and Dymicron Inc. and has received less than $10,000 in remuneration in the past 2 years. This consulting activity was not related to the subject of the manuscript. None of the other authors have competing interests to declare.

# REFERENCES

1 Lopes SV, Collins MN, Reis RL, Oliveira JM, Silva-Correia J. Vascularization approaches in tissue engineering: recent developments on evaluation tests and modulation. *ACS Appl Bio Mater*. 2021; **4**: 2941- 2956. doi:10.1021/acsabm.1c00051

2 White SM, Pittman CR, Hingorani R, et al. Implanted cell-dense prevascularized tissues develop functional vasculature that supports reoxygenation after thrombosis. *Tissue Eng Part A*. 2014; **20**: 2316- 2328. doi:10.1089/ten.TEA.2013.0311

3 Mastrullo V, Cathery W, Velliou E, Madeddu P, Campagnolo P. Angiogenesis in tissue engineering: as nature intended? *Front Bioeng Biotechnol*. 2020; **8**:1-13. doi:10.3389/fbioe.2020.00188

4 Masson-Meyers DS, Tayebi L. Vascularization strategies in tissue engineering approaches for soft tissue repair. *J Tissue Eng Regen Med*. 2021; **15**: 747- 762. doi:10.1002/term.3225

5 Xiao X, Wang W, Liu D, et al. The promotion of angiogenesis induced by three-dimensional porous beta-tricalcium phosphate scaffold with different interconnection sizes via activation of PI3K/Akt pathways. *Sci Rep*. 2015; **5**: 9409. doi:10.1038/srep09409

6 Kniebs C, Kreimendahl F, Köpf M, Fischer H, Jockenhoevel S, Thiebes AL. Influence of different cell types and sources on pre-vascularisation in fibrin and agarose-collagen gels. *Organogenesis*. 2020; **16**: 14- 26. doi:10.1080/15476278.2019.1697597

7 Sarker M, Chen XB, Schreyer DJ. Experimental approaches to vascularisation within tissue engineering constructs. *J Biomater Sci Polym Ed*. 2015; **26**: 683- 734. doi:10.1080/09205063.2015.1059018

8 Min S, Ko IK, Yoo JJ. State-of-the-art strategies for the vascularization of three-dimensional engineered organs. *Vasc Specialist Int*. 2019; **35**: 77- 89. doi:10.5758/vsi.2019.35.2.77

9 Masson-Meyers DS, Bertassoni LE, Tayebi L. Oral mucosa equivalents, prevascularization approaches, and potential applications. *Connect Tissue Res*. 2022; **1-16**: 514- 529. doi:10.1080/03008207.2022.2035375

10 Costa-Almeida R, Granja PL, Soares R, Guerreiro SG. Cellular strategies to promote vascularisation in tissue engineering applications. *Eur Cell Mater*. 2014; **28**: 51- 66; discussion 66–57. doi:10.22203/ecm.v028a05

11 Baiguera S, Ribatti D. Endothelialization approaches for viable engineered tissues. *Angiogenesis*. 2013; **16**: 1- 14. doi:10.1007/s10456-012-9307-8

12 Um Min Allah N, Berahim Z, Ahmad A, Kannan TP. Biological interaction between human gingival fibroblasts and vascular endothelial cells for angiogenesis: a co-culture perspective. *Tissue Eng Regen Med*. 2017; **14**: 495- 505. doi:10.1007/s13770-017-0065-y

13 El-Sherbiny IM, Yacoub MH. Hydrogel scaffolds for tissue engineering: progress and challenges. *Glob Cardiol Sci Pract*. 2013; **2013**: 316- 342. doi:10.5339/gcsp.2013.38

14 Dong C, Lv Y. Application of collagen scaffold in tissue engineering: recent advances and new perspectives. *Polymers (Basel)*. 2016; **8**:1-20. doi:10.3390/polym8020042

15 Yue B. Biology of the extracellular matrix: an overview. *J Glaucoma*. 2014; **23**: S20- S23. doi:10.1097/IJG.0000000000000108

16 Wang Y, Kankala RK, Ou C, Chen A, Yang Z. Advances in hydrogel-based vascularized tissues for tissue repair and drug screening. *Bioactive Mater*. 2022; **9**: 198- 220. doi:10.1016/j.bioactmat.2021.07.005

17 Malda J, Visser J, Melchels FP, et al. 25th anniversary article: engineering hydrogels for biofabrication. *Adv Mater*. 2013; **25**: 5011- 5028. doi:10.1002/adma.201302042

18 Benning L, Gutzweiler L, Tröndle K, et al. Assessment of hydrogels for bioprinting of endothelial cells. *J Biomed Mater Res A*. 2018; **106**: 935- 947. doi:10.1002/jbm.a.36291

19 Dash BC, Duan K, Xing H, Kyriakides TR, Hsia HC. An in situ collagen-HA hydrogel system promotes survival and preserves the proangiogenic secretion of hiPSC-derived vascular smooth muscle cells. *Biotechnol Bioeng*. 2020; **117**: 3912- 3923. doi:10.1002/bit.27530

20 Kreimendahl F, Ossenbrink S, Köpf M, et al. Combination of vascularization and cilia formation for three-dimensional airway tissue engineering. *J Biomed Mater Res A*. 2019; **107**: 2053- 2062. doi:10.1002/jbm.a.36718

21 Massa S, Sakr MA, Seo J, et al. Bioprinted 3D vascularized tissue model for drug toxicity analysis. *Biomicrofluidics*. 2017; **11**:044109. doi:10.1063/1.4994708

22 Mazio C, Casale C, Imparato G, et al. Pre-vascularized dermis model for fast and functional anastomosis with host vasculature. *Biomaterials*. 2019; **192**: 159- 170. doi:10.1016/j.biomaterials.2018.11.018

23 Montaño I, Schiestl C, Schneider J, et al. Formation of human capillaries in vitro: the engineering of prevascularized matrices. *Tissue Eng Part A*. 2010; **16**: 269- 282. doi:10.1089/ten.TEA.2008.0550

24 Entezar-Almahdi E, Heidari R, Ghasemi S, Mohammadi-Samani S, Farjadian F. Integrin receptor mediated pH-responsive nano-hydrogel based on histidine-modified poly(aminoethyl methacrylamide) as targeted cisplatin delivery system. *J Drug Deliv Sci Technol*. 2021; **62**:102402. doi:10.1016/j.jddst.2021.102402

25 Ghasemi S, Owrang M, Javaheri F, Farjadian F. Spermine modified PNIPAAm nano-hydrogel serving as thermo-responsive system for delivery of cisplatin. *Macromol Res*. 2022; **30**: 314- 324. doi:10.1007/s13233-022-0035-7

26 Lin K, Zhang D, Macedo MH, Cui W, Sarmento B, Shen G. Advanced collagen-based biomaterials for regenerative biomedicine. *Adv Funct Mater*. 2019; **29**:1804943. doi:10.1002/adfm.201804943

27 Sarrigiannidis SO, Rey JM, Dobre O, González-García C, Dalby MJ, Salmeron-Sanchez M. A tough act to follow: collagen hydrogel modifications to improve mechanical and growth factor loading capabilities. *Mater Today Bio*. 2021; **10**:100098. doi:10.1016/j.mtbio.2021.100098

28 Catoira MC, Fusaro L, Di Francesco D, Ramella M, Boccafoschi F. Overview of natural hydrogels for regenerative medicine applications. *J Mater Sci Mater Med*. 2019; **30**:115. doi:10.1007/s10856-019-6318-7

29 Chan EC, Kuo SM, Kong AM, et al. Three dimensional collagen scaffold promotes intrinsic vascularisation for tissue engineering applications. *PLoS One*. 2016; **11**:e0149799. doi:10.1371/journal.pone.0149799

30 Groger A, Megas IF, Noah EM, Pallua N, Grieb G. Proliferation of endothelial cells (HUVEC) on specific-modified collagen sponges loaded with different growth factors. *Int J Artif Organs*. 2021; **44**: 880- 886. doi:10.1177/03913988211043198

31 Parthiban SP, He W, Monteiro N, Athirasala A, França CM, Bertassoni LE. Engineering pericyte-supported microvascular capillaries in cell-laden hydrogels using stem cells from the bone marrow, dental pulp and dental apical papilla. *Sci Rep*. 2020; **10**:21579. doi:10.1038/s41598-020-78176-7

32 Samal J, Weinandy S, Weinandy A, et al. Co-culture of human endothelial cells and foreskin fibroblasts on 3D silk-fibrin scaffolds supports vascularization. *Macromol Biosci*. 2015; **15**: 1433- 1446. doi:10.1002/mabi.201500054

33 Heller M, Bauer HK, Schwab R, et al. The impact of intercellular communication for the generation of complex multicellular prevascularized tissue equivalents. *J Biomed Mater Res A*. 2020; **108**: 734- 748. doi:10.1002/jbm.a.36853

34 Lee J, Shin D, Roh JL. Use of a pre-vascularised oral mucosal cell sheet for promoting cutaneous burn wound healing. *Theranostics*. 2018; **8**: 5703- 5712. doi:10.7150/thno.28754

35 Tabatabaei F, Rasoulianboroujeni M, Yadegari A, Tajik S, Moharamzadeh K, Tayebi L. Osteo-mucosal engineered construct: in situ adhesion of hard-soft tissues. *Mater Sci Eng C*. 2021; **128**:112255. doi:10.1016/j.msec.2021.112255

36 Dongari-Bagtzoglou A, Kashleva H. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. *Nat Protoc*. 2006; **1**: 2012- 2018. doi:10.1038/nprot.2006.323

37 Almela T, Brook IM, Moharamzadeh K. Development of three-dimensional tissue engineered bone-oral mucosal composite models. *J Mater Sci Mater Med*. 2016; **27**: 65. doi:10.1007/s10856-016-5676-7

38 Tabatabaei F, Moharamzadeh K, Tayebi L. Fibroblast encapsulation in gelatin methacryloyl (GelMA) versus collagen hydrogel as substrates for oral mucosa tissue engineering. *J Oral Biol Craniofacial Res*. 2020; **10**: 573- 577.

39 Monteiro N, He W, Franca CM, Athirasala A, Bertassoni LE. Engineering microvascular networks in LED light-cured cell-laden hydrogels. *ACS Biomater Sci Eng*. 2018; **4**: 2563- 2570. doi:10.1021/acsbiomaterials.8b00502

40 Byfield FJ, Reen RK, Shentu TP, Levitan I, Gooch KJ. Endothelial Actin and cell stiffness is modulated by substrate stiffness in 2D and 3D. *J Biomech*. 2009; **42**: 1114- 1119. doi:10.1016/j.jbiomech.2009.02.012

41 McFadden TM, Duffy GP, Allen AB, et al. The delayed addition of human mesenchymal stem cells to pre-formed endothelial cell networks results in functional vascularization of a collagen-glycosaminoglycan scaffold in vivo. *Acta Biomater*. 2013; **9**: 9303- 9316. doi:10.1016/j.actbio.2013.08.014

42 Rasoulianboroujeni M, Kiaie N, Tabatabaei FS, et al. Dual porosity protein-based scaffolds with enhanced cell infiltration and proliferation. *Sci Rep*. 2018; **8**:14889. doi:10.1038/s41598-018-33245-w

43 Kosyakova N, Kao DD, Figetakis M, et al. Differential functional roles of fibroblasts and pericytes in the formation of tissue-engineered microvascular networks in vitro. *NPJ Regen Med*. 2020; **5**:1. doi:10.1038/s41536-019-0086-3

44 Cheung JW, Jain D, McCulloch CA, Santerre JP. Pro-angiogenic character of endothelial cells and gingival fibroblasts cocultures in perfused degradable polyurethane scaffolds. *Tissue Eng Part A*. 2015; **21**: 1587- 1599. doi:10.1089/ten.TEA.2014.0548