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Abstract: Collagen scaffolds provide a promising platform for tissue regeneration due to their ability to support cell attachment, proliferation and differentiation. In this study, we used porous freeze-dried collagen scaffolds with either uniaxially-aligned or randomly-oriented pores to investigate the formation of vascular structures in vitro. We characterised the scaffold pore structure, specific permeability, cell migration and endothelial cell self-assembly into vessel-like networks in mono- and co-culture with osteoblasts. Our work shows that improved pre-vascularisation can be achieved in uniaxially-aligned scaffolds and in a co-culture environment.

Collagen scaffolds with tailored pore geometry for building 3-dimensional vascular networks

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Abstract

Collagen scaffolds provide a promising platform for tissue regeneration due to their ability to support cell attachment, proliferation and differentiation. In this study, we used porous freeze-dried collagen scaffolds with either uniaxially-aligned or randomly-oriented pores to investigate the formation of vascular structures *in vitro*. We characterised the scaffold pore structure, specific permeability, cell migration and endothelial cell self-assembly into vessel-like networks in mono- and co-culture with osteoblasts. Our work shows that improved pre-vascularisation can be achieved in uniaxially-aligned scaffolds and in a co-culture environment.

Keywords:

Biomaterials

Porous materials

Pore Structure

Specific permeability

Vascularization

Collagen

1. Introduction

Production of engineered tissues with clinically-relevant dimensions requires establishment of perfusable vasculature to ensure effective nutrient and waste product exchange throughout the tissue construct [1]. One pre-vascularization approach employs self-assembly of endothelial cells (ECs) and supporting cell populations into vessel-like structures within 3-dimensional (3D) matrices. It has been shown that using osteoblasts (HObs) as supportive cells in co-culture with ECs facilitates extensive and stable microvessel-like network formation both *in vitro* and *in vivo*, due to direct and indirect intercellular communication [2, 3].

The scaffold pore structure has been shown to affect cell migration, proliferation, differentiation and tissue formation *in vitro* and *in vivo* [4-6]. In the present study, we manipulated the scaffold pore geometry by varying the freeze-drying conditions [7], and used the pores as channels for vascular self-assembly. We fabricated isotropic and anisotropic collagen scaffolds, with randomly-oriented and uniaxially-aligned pores respectively, and assessed the effect of scaffold pore structure on vessel-like network formation, in EC mono-culture and in co-culture with HObs.

2. Experimental

2.1 Scaffold fabrication

All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Two types of collagen scaffolds were produced: isotropic and anisotropic (Fig. 1). Collagen slurry was formed using type 1 bovine collagen in 0.05 M acetic acid (1% w/v), homogenised for 20 min, 15000 rpm and centrifuged at 2500 rpm for 5 min. The suspension was transferred into either PTFE or PTFE/copper moulds and then freeze-dried at a cooling rate of 0.9°C/min up to a final freezing temperature of -40°C, held for 1 h to ensure complete solidification and then sublimed under 50 mThorr at 0°C for 24 h. For cell experiments, the scaffolds were cross-linked at a ratio of 2.5:1:1 of EDC:NHS:carboxylic collagen acid groups for 4 h, washed in PBS, sterilized in 70% ethanol and washed thrice in PBS for 20 min.

Figure 1: Schematic representation of scaffold production. A one-component mould promotes multidirectional heat transfer producing isotropic scaffolds with randomly oriented pores. A two-component mould, promotes heat transfer from bottom to top due to thermal conductivity mismatch ($k_{Cu}/k_{PTFE} \sim 1600$), producing anisotropic scaffolds with unidirectionally aligned pores.

2.2 Scaffold pore structure and specific permeability

Scaffolds were cut along the longitudinal direction, platinum-sputtered and imaged using a Jeol-5800 scanning electron microscope. For surface area measurements, scans with 2.32 μm linear resolution were acquired using a Zeiss Xradia 510 Versa 3D X-ray microscope. The source voltage, current and angular steps were 50 kV, 80 μA and 0.36°, respectively. X-ray images were processed using a

customized Matlab program described by Varley et al. [9]. 15 sub-volumes of $400 \times 400 \times 400 \mu\text{m}$ were analysed per sample. Through-thickness specific permeability was measured by using Darcy's Law with a constant pressure gradient method as described previously [9]. The pressure gradients created in the scaffolds (5 mm diameter, 4 mm height) were $\sim 60 \text{ Pa} \cdot \text{mm}^{-1}$. Scaffolds were hydrated and then mounted on a 4.8 mm diameter aperture.

2.3. Cell migration and microvessel-like formation

Scaffolds were seeded with either Human Umbilical Cord ECs (HUVECs, Public Health England) alone or in co-culture with HObs (Public Health England) at a ratio of 4:1. A 75 μL droplet of media containing 3×10^5 cells was placed on top of each scaffold, located on hydrophobic PTFE membrane, and incubated under static conditions at 37 °C for 4 h to allow cell attachment. Cell-seeded constructs were kept in either HUVECs growth medium (EBM-2 supplemented with EGM-2 bullet kit (Lonza)), or in a 4:1 mixture of HUVEC:HObs growth medium (McCoy's 5A medium (Fisher Scientific), supplemented with 100 U/ml Penicillin-Streptomycin (Life Technologies), 30 mg/mL Vitamin C salt (L-Ascorbic acid, Insight Biotechnology) and 5% (v/v) FBS). Cell medium was changed every other day. At days 1 and 14, the cellular constructs were fixed in 4% (w/v) paraformaldehyde, followed by permeabilization and blocking with 0.5% Triton X-100/1% BSA solution for 1 h. Then the scaffolds were incubated with mouse anti-human anti CD-31 antibody (Abcam) and Phalloidin for 1 h, followed by incubation with goat anti-mouse Alexa Fluor 488 antibody (Life Technologies) for 2 h and mounting in DAPI-Fluoroshield for 10 min. After each step, the samples were washed thrice with PBS/0.05% Tween-20 for 10 min. For cell infiltration studies, only DAPI staining was used. All samples were imaged using an LSM 710 Zeiss confocal microscope.

3. Results and discussion

Scaffold design is crucial for numerous tissue engineering applications as pore content, interconnectivity, orientation and shape determine the availability of nutrients, oxygen and material

surface for cell attachment, thus affecting cell morphology, infiltration and subsequent tissue formation.

Fig. 2a shows the anisotropic scaffolds fabricated in this study and their interconnected pores aligned parallel to ice growth direction. The isotropic scaffolds have non-equiaxed randomly distributed pores.

Figure 2: (a) Representative SEM images showing longitudinal cross-sections of the two scaffolds at low (top) and high (bottom) magnifications. (b) Fluid flow rate as a function of time.

Permeability depends strongly on porosity and pore structure [8], affecting diffusion and thus the scaffold performance. Fig. 2b shows that the flow rate in both scaffolds decreases slightly with time and reaches a plateau after 40-45 sec. The average values for the through-thickness specific permeability of the anisotropic and isotropic scaffolds are $7.76 \times 10^{-11} \pm 0.36 \times 10^{-11} \text{ m}^2$ and $2.09 \times 10^{-11} \pm 0.25 \times 10^{-11} \text{ m}^2$ respectively ($p < 0.05$), which are in agreement with previous studies on similar scaffolds [9,10]. Table 1 shows that anisotropic scaffolds have a slightly larger equivalent pore diameter and are less porous. This suggests that there are fewer pores per unit volume compared to the isotropic scaffolds. Indeed, 3D X-ray microscopy showed ~25% more pores in the isotropic scaffolds compared to the anisotropic ones. In addition, anisotropic scaffolds have a higher specific surface area, albeit with lower porosity levels, which

is attributed to differences in pore shape. Isotropic scaffolds have less elongated pores and smoother pore walls than the anisotropic ones, resulting in a lower specific surface area. The higher through-thickness permeability of the anisotropic scaffolds is attributed to the aligned and more interconnected pore structure. A pore interconnectivity parameter can be estimated from the product of the specific permeability and the specific surface area giving values of 4.1 and 0.8 μm for the anisotropic and isotropic scaffolds, respectively. Since permeability drops and specific surface area rises as the scale of the structure becomes finer, the pore interconnectivity parameter is scale independent, at least to a first approximation.

Fig. 3a shows that the aligned pore structure of the anisotropic scaffolds promoted more efficient cell infiltration, and hence a more uniform through-thickness cell distribution. In contrast, cells were preferentially located on the seeded surface of the isotropic scaffolds, creating a multilayer sheet (~300-600 μm deep). Self-assembly of endothelial cells into microvessel-like structures was also structure-dependent (Fig. 3b-c). In the isotropic scaffolds, ECs retained a cobblestone morphology when cultured alone, whereas a co-culture with HOBs resulted in an increase in CD31 expression by ECs, evident both at the scaffold surface and mid-cross-sections. Although co-cultured cells showed some degree of self-assembly into multinucleated structures on the scaffold surface, isolated cells with no directionality can be seen on the mid-cross-sections (Fig. 3c). In the anisotropic scaffolds, ECs in monoculture produced elongated multinucleated structures aligned along the interconnected pores, both on top and in the mid-cross-sections, replicating the directionality of the anisotropic pore structure. When co-cultured with HOBs, the endothelial self-assembly was further enhanced. Our findings suggest that highly permeable scaffolds, with aligned pores mimicking (to some extent) the tubular structure of larger blood vessels, can facilitate uniform cell seeding and promote the formation of vessel-like networks throughout the scaffold, presumably by improving mass transfer for cell growth and function. Further investigation into the pre-vascularization mechanisms as a function of pore structure is required.

Figure 3: (a) Representative longitudinal cross-sections showing EC distribution within scaffolds (Day 1).
(b) Top views and (c) mid-cross-sections showing ECs in mono- and co-culture with HObs (Day 14).
Endothelial cell marker CD31 shown in green, actin in red and cell nuclei in blue.

4. Conclusions

We have shown that scaffold pore structure affects endothelial cell distribution, morphology and self-organisation when cultured alone and in co-culture with osteoblasts, and that co-culture conditions improve cell organisation into vessel-like structures. The anisotropic scaffolds, due to their aligned pore structure and high pore interconnectivity, facilitated fluid perfusion and promoted a more uniform cell distribution, which in turn supported better intercellular communication and vessel-like network formation.

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	Mean Equivalent Diameter (μm)	Specific Surface Area (10^4 m^{-1})	Porosity (%)
ISO	121.4 ± 5.9	4.0 ± 0.7 *	93.1 ± 0.3 *
ANI	132.1 ± 16.6	5.3 ± 0.4	88.1 ± 0.8

Table 1: Mean equivalent diameter (>900 pores were analysed), specific surface area (scaffold surface area/ scaffold volume) and porosity obtained using 3D X-ray microscopy. Data are presented as the mean $\pm$ standard deviation. * $p < 0.05$ vs anisotropic scaffolds.

Figure 1

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Figure 2
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Figure 3
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	Median age (years) (interquartile range)	Median duration of disease (years) (interquartile range)	Median duration of disease (years) (interquartile range)
1000	55 (45-65)	10 (5-15)	10 (5-15)
500	55 (45-65)	10 (5-15)	10 (5-15)