Fabrication of a self-assembled honeycomb nanofibrous scaffold to guide endothelial morphogenesis

Citation for published version (APA):

Yao, T., Wieringa, P. A., Chen, H., Amit, C., Samal, P., Giselsbrecht, S., Baker, M. B., & Moroni, L. (2020). Fabrication of a self-assembled honeycomb nanofibrous scaffold to guide endothelial morphogenesis. *Biofabrication*, 12(4), [045001]. <https://doi.org/10.1088/1758-5090/ab9988>

Document status and date:

Published: 01/10/2020

DOI:

[10.1088/1758-5090/ab9988](https://doi.org/10.1088/1758-5090/ab9988)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

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PAPER

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To cite this article: Tianyu Yao *et al* 2020 *Biofabrication* **12** 045001

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PAPER

Fabrication of a self-assembled honeycomb nanofibrous scaffold to guide endothelial morphogenesis

RECEIVED
14 January 2020REVISED
12 May 2020ACCEPTED FOR PUBLICATION
4 June 2020PUBLISHED
7 July 2020Tianyu Yao¹ , Paul Andrew Wieringa¹ , Honglin Chen², Chandrakar Amit¹, Pinak Samal¹, Stefan Giselbrecht¹ , Matthew B Baker¹ and Lorenzo Moroni^{1,3} ¹ Complex Tissue Regeneration Department, MERLN Institute for Technology Inspired Regenerative Medicine, Maastricht University, Maastricht 6229 ER, The Netherlands² Institute for Life Science, School of Medicine, South China University of Technology, Guangzhou 510640, People's Republic of ChinaE-mail: l.moroni@maastrichtuniversity.nl**Keywords:** electrospinning, honeycomb, nanofibrous, angiogenesisSupplementary material for this article is available [online](#)**Abstract**

Controlling angiogenesis within tissue engineered constructs remains a critical challenge, especially with regard to the guidance of pre-vascular network formation. Here, we aimed to regulate angiogenesis on a self-assembled honeycomb nanofibrous scaffold. Scaffolds with honeycomb patterns have several desirable properties for tissue engineering, including large surface area, high structural stability and good permeability. Furthermore, the honeycomb pattern resembles early vascular network formation. The self-assembly electrospinning approach to honeycomb scaffolds is a technically simple, rapid, and direct way to realize selective deposition of nanofibers. To evaluate cell compatibility, spreading, proliferation and tube formation, human umbilical vein endothelial cells (HUVECs) were cultured on honeycomb scaffolds, as well as on random scaffolds for comparison. The optimized honeycomb nanofibrous scaffolds were observed to better support cell proliferation and network formation, which can facilitate angiogenesis. Moreover, HUVECs cultured on the honeycomb scaffolds were observed to reorganize their cell bodies into tube-like structures containing a central lumen, while this was not observed on random scaffolds. This work has shown that the angiogenic response can be guided by honeycomb scaffolds, allowing improved early HUVECs organization. The guided organization via honeycomb scaffolds can be utilized for tissue engineering applications that require the formation of microvascular networks.

1. Introduction

With significant progress in tissue engineering over recent years, there comes a great promise to develop functional constructs resembling the structural organization of native tissues to repair or replace damaged tissues or organs [1, 2]. Yet, angiogenesis remains one of the most challenging issues [3, 4]. During formation of new tissue, the vessels created by the natural angiogenic process is required to supply oxygen and nutrition for the regenerating tissues as well as to remove metabolic waste products [5–7]. Lack of efficient vascularization limits the size of tissue-engineered constructs [1, 8], and often is the reason for failure of translation. Implantation of tissue constructs in a poorly vascularized site often leads

to lack of tissue integration and cell death [9, 10]. As a result, many tissue-engineered constructs also fail *in vivo* [1, 11, 12]. The angiogenesis process is critical for the successful regeneration of most tissues within the human body [13, 14].

Numerous studies have been conducted to improve angiogenesis in tissue engineering [6, 7]. Recent strategies can be classified into three categories: (1) scaffold design [15–18], (2) growth factor delivery [19, 20] and (3) prevascularization [21–23]. Tissue-engineered scaffolds have been considered to be a common and simple strategy to induce angiogenesis [7]. An engineered scaffold should typically provide well-defined biochemical (e.g. surface chemistry [24]) and biophysical cues (e.g. fibrous structure [25], hydrophilicity [26] and stiffness [27]) to mimic the environment of a cell's native extracellular matrix (ECM). Three techniques have proven successful

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to fabricate the biomimetic nanofibrous structure: molecular self-assembly [28–31], phase separation [32, 33], and electrospinning [34–36]. Electrospinning is a simple and cost-effective processing technique widely used to produce nanofibers for tissue engineering applications [2]. Several fascinating bio-inspired nanofibrous structures have been successfully fabricated by electrospinning such as a lotus leaf [37], a silver ragwort leaf [38], a feather [39], a plant tendril [40], a spider web [41], polar bear hair [42], and a honeycomb [43]. The honeycomb-patterned nanofibrous structures have interesting mechanical properties for tissue-engineering, and can be fabricated by the self-assembly method of electrospinning [43–45]. By adjusting the electrospinning parameters, a well-organized honeycomb structure with different diameters of the honeycomb cells can be obtained [43, 45]. Previously, honeycomb-patterned 2D films, fabricated by casting the polymer solution under moist air, was shown to be a suitable substrate to enhance cell adhesion and proliferation [46, 47]. Honeycomb 3D scaffolds have a large surface area, high porosity and favorable mechanical performance, which provide them great potential as porous scaffolds for tissue engineering [48–51].

Blood vessels develop via two consecutive processes, vasculogenesis and angiogenesis [52]. During

vasculogenesis, formation of the earliest vessels is achieved. It is reported that fusion of blood-islands leads to the vasculogenesis of honeycomb-shaped primary capillaries [53]. While in angiogenesis, new blood vessels derive from already existing vessels [52]. Angiogenesis involves the proliferation, migration, and remodeling of endothelial cells in the process of tube formation. During angiogenesis, endothelial cells can form honeycomb-like network which facilitate tube formation [54, 55]. The presence of a honeycomb-like vascular structure indicates that vasculogenesis and the initial steps of angiogenesis proceeded normally [56]. Therefore, honeycomb network of endothelial cells is a very important morphogenetic trait and can be used to indicate both vasculogenesis and angiogenesis.

Previous studies have shown that endothelial cell morphogenesis into capillary-like structures can be induced and guided by the geometrical distribution of materials [14, 57]. One study showed that endothelial cells cultured on substrates micropatterned with 10 μm -wide lines of fibronectin formed capillary tube-like structures containing a central lumen; cells cultured on wider (30 μm) lines did not organize into tubes [57]. Moon and colleagues micropatterned PEGDA hydrogels with RGDS in different geometries [14]. As a result, endothelial cells cultured on RGDS

patterns reorganized their cell bodies into tube-like structures on 50- μm -wide stripes, but not on wider stripes. These results suggested that endothelial cell morphogenesis can be regulated by geometrical cues. Development of a well-designed scaffold in which capillary tubes consistently form is an important step toward the fabrication of engineered tissues. In this article, we fabricated a nanofibrous scaffold with a unique honeycomb pattern as a biocompatible template to induce the formation of honeycomb-like vascular network. Honeycomb patterns were obtained by the self-assembly of electrospun fibers, and cell culture tests were performed using human umbilical vein endothelial cells (HUVECs). Both monoculture and co-culture with human mesenchymal stromal cells (hMSCs) were used in order to investigate their angiogenic ability on these scaffolds.

2. Materials and methods

2.1. Fabrication of honeycomb nanofibrous scaffolds

The electrospinning process used in this study is a homemade electrospinning set-up as mentioned before [58]. Poly(ϵ -caprolactone) with different molecular weight (PCL; $M_n = \sim 45\,000$ and $\sim 80\,000\text{ g mol}^{-1}$) were purchased from Sigma-Aldrich, as also used in our previous studies for fabrication of honeycomb nanofibrous scaffolds [45]. Briefly, PCL was placed in a mixture of chloroform:dimethylformamide (4:1) at a certain concentration (table S1 (available online at stacks.iop.org/BF/12/045001/mmedia)) and then stirred overnight at room temperature before electrospinning to ensure good polymer solubilization. The polymer solution was then filled into a 5 mL syringe (BD biosciences) and delivered to the stainless steel needle (Unimed S.A.; 0.8 mm) via a PTFE tube (Sigma-Aldrich). This steel nozzle was connected with a high voltage supplier to generate the electric field strength during electrospinning. Flow rate of polymer solution was controlled by a syringe pump (Harvard). An aluminum foil collector plate was used to collect fibers under the jet. The distance between the tip of the nozzle and the collecting plate was referred as the working distance (table S1). Honeycomb nanofibrous scaffolds were obtained by a self-assembly electrospinning approach, which was only controlled by the electrospinning parameter. Therefore, no additional equipment was required during electrospinning. Electrospinning parameter for the fabrication of random and honeycomb scaffolds are described in table S1. The experiments were done at 25 °C and the humidity was kept around 35%.

2.2. Characterization of honeycomb structures

The fiber structure and pattern of honeycomb scaffolds were detected by a scanning electron microscopy (Philips XL30 ESEM). Before observation, the

scaffolds were pretreated with gold by using sputter coater 108 Auto set at 30 mA for 80 s. The honeycomb diameter and size were analyzed with Image J software. The nanofiber alignment into the honeycomb pattern was quantified by an Image J plugin (called Orientation J) to indicate the orientation coherency. The confocal laser scanning microscopy (Keyence) was used to measure the depth of both random and honeycomb electrospun scaffolds.

2.3. Cell culture and seeding

HUVECs were provided by Lonza and incubated in endothelial growth medium (EGM, Lonza) which includes endothelial basal medium (EBM) and EGMTM-2 SingleQuotsTM supplements. For experiments, cells were used between passages 4 and 7. The tube formation assay was performed as Lonza described in instructions. Briefly, Matrigel[®] (Corning) was used to coat the wells of 48-well plates (0.15 ml per well) and was left to incubate at 37 °C for 30 min. After incubation, HUVECs were seeded on each well at the density of 50 000 cells cm^{-2} in EGM. The tube formation of cells was visualized using a light microscope. As showed in figures 1(e)–(f), HUVECs can form the capillary network after 16 h, which was also reported by Lonza. Prior to seeding cells, all PCL electrospun scaffolds were cut into round films (diameter = 15 mm), and washed in distilled water to remove the residues from sample preparation, further sterilized by 70% ethanol solution for 30 min, and dried in a biosafety cabinet. In order to increase cell attachment, the scaffolds were coated using Matrigel[®] (1:150 dilution in EGM) overnight. HUVECs were seeded on each random and honeycomb electrospun scaffold with a density of 20 000 cells cm^{-2} . Then the cells were cultured in EGM for 1, 3, and 5 d in an incubator at 37 °C.

2.4. Coculture and cells seeding

hMSCs (D8011 L, Texas A&M University) were incubated in basic medium containing α -MEM (Gibco) with 10% FBS (Sigma), 0.2 mM ascorbic acid (Sigma), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Gibco). hMSCs and HUVECs were co-cultured at a ratio of 1:1 on both scaffolds. The seeding density was kept at 20 000 cells cm^{-2} . Cells were then co-cultured in a mixed media composed of EGM and basic medium at 1:1 ratio for 5 d.

2.5. Live/dead assay

Cell cytotoxicity was evaluated by a live/dead assay kit (Fisher Scientific) after 1 d of cells cultured on electrospun scaffolds. Briefly, 1 μM of Calcein AM and 6 μM of ethidium homodimer-1 (EthD-1) were dissolved in 200 μl PBS and then added into cell-scaffolds, followed with incubation for 30 min at 37 °C. Calcein AM was stained by live cells; EthD-1 only stained dead cells. After washing with PBS,

Figure 1. SEM images of random (a) and (b) and honeycomb (c) and (d) PCL electrospun fibers. Images (e) and (f) showed the tube formation of HUVECs cultured on Matrigel-coated wellplate after 16 h. Scale bars are 500 μm (a) and (c) and 100 μm (b), (d), (e) and (f).

the cells were imaged by using a fluorescence microscopy (Nikon Eclipse Ti-S). Live and dead cells were quantified from live/dead assay images. The percentage of live cells was counted by an equation ($\text{Viable cells \%} = \text{Live cells} / \text{Total cells} \times 100$).

2.6. Cell viability and proliferation

The viability of HUVECs on electrospun scaffolds was determined using the PrestoBlue kit (Thermo Fisher Scientific). Briefly, the cell-scaffolds were immersed in warm PBS and treated with PrestoBlue medium (PrestoBlue: EGM = 1:9) in an incubator. After 30 min incubation, 100 μl of PrestoBlue medium was added into a black 96 well plate with a clear bottom. The fluorescence of each solution was calculated by using a microplate reader (CLARIO star, BMG Labtech) at 590 nm.

Cell proliferation was assessed after 1, 3, and 5 d culture by using a CyQUANT™ Cell Proliferation Assay Kit (Fisher Scientific). The samples were frozen and thawed three times. 250 μl of Proteinase K in Tris/EDTA solution was then added to each sample. After overnight incubation at 56 °C, 40 μl of digested samples was placed into a black 96 well plate and the same volume of lysis buffer was added into the well plate for 1 h incubation. Finally, GR dye (80 μl) was transferred into each well and protected in dark for 15 min. Fluorescence of the solution was measured with the plate reader (CLARIO star, BMG Labtech) at emission and excitation wavelengths of 480 and 520 nm, respectively.

2.7. Immunofluorescence staining

HUVECs were cultured on the random and honeycomb scaffolds for 5 d and then fixed using 4% formaldehyde for 30 min. Fixed samples were washed with PBS for 3 times. After washing, samples were permeabilized with 0.1% Triton-X 100 in PBS for 15 min and blocked in blocking buffer (including 0.05% Tween 20, 5% goat serum, and 1% BSA in PBS) for 1 h. The cell-scaffolds were soaked in 200 μl primary antibodies (anti-CD31 or anti-VE-Cadherin antibody, 1:200 dilution in blocking solution) overnight at 4 °C after blocking. The incubated samples were immersed in 200 μl wash buffer (blocking buffer without goat serum), then placed in secondary antibody solution (goat-anti mouse; Alexa Fluor 488; 1:200 dilution in wash buffer). After 1 h of incubation with secondary antibody, the samples were cleaned by PBS three times. Cell nuclei were labeled with DAPI for 5 min, followed by washing with PBS. Finally cells on scaffolds were observed with a fluorescent microscope (Nikon Eclipse Ti-S). To quantify angiogenesis, Image J was used to analyze the length of tube formation. A confocal laser excitation microscope (TCS SP8 STED, Leica) was used to access images of HUVECs on scaffolds in different z-stages. Image of the single tube was consisted of 71 individual z-axis stacks. Z-stack images were generated by scanning every 0.15 μm in thickness. Huygens software (Scientific Volume Imaging, Hilversum, NL) was employed for three-dimensional (3D) reconstruction of confocal images and for analysis of the hollow lumen of HUVECs on honeycomb scaffolds.

Figure 2. The diameter (a) and area (b) distribution of honeycomb patterns in nanofibrous scaffolds. Images (c) and (d) are optical profilometer scans of a honeycomb pattern. (e) Profile graph measuring the height of honeycomb structure in the nanofibrous scaffolds.

2.8. Cell morphology and attachment

SEM images of HUVECs seeded on honeycomb electrospun scaffolds after 5 d was performed. In brief, samples were cleaned with PBS three times after fixation with 4% formaldehyde. The fixed samples were dehydrated by a standard ethanol series (30%, 50%, 70%, 80%, 90%, 96% and 100%) for at least 15 min. The dehydrated samples were immersed in hexamethyldisilazane (HMDS; Sigma-Aldrich) for 15 min twice and then dried overnight. After coating with gold, samples were observed by SEM. Cell morphology and organization on the fibrous scaffolds were also evaluated by using phalloidin (Invitrogen) to label F-actin filaments. After fixation, the cells-scaffolds were incubated in DAPI (Invitrogen, 1:200 in PBS) and Alexa Fluor 568 phalloidin (1:200 in PBS) solution separately according to manufacturer's instructions. Cell were immersed in PBS three times in order to wash extra fluorescence and finally imaged

using the fluorescence microscopy and a confocal laser excitation microscope (TCS SP8 STED, Leica).

2.9. Statistical analysis

Statistical differences were assessed by using Graph-Pad Prism 7 software. Unpaired t-test was used to compare the significance between two groups. In all cases, different significance were shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results and discussion

Only a few studies have shown the effect of geometrical control on morphogenesis of ECs into tubular structures by using micropatterned stripes substrates [14, 57]. In the present study, we analyzed the effect of honeycomb patterns on angiogenesis using a self-assembled honeycomb scaffolds. To provide

structural guidance for cell response, PCL nanofibrous scaffolds with random and honeycomb morphologies were fabricated.

3.1. Morphology of random and honeycomb electrospun scaffolds

The morphologies of random and honeycomb electrospun scaffolds were examined by scanning electron microscopy (SEM). SEM images clearly showed a difference in morphology between two types of scaffolds. As shown in figures 1(a)–(b), PCL random fibrous scaffolds were formed with randomly oriented and uniform fibers. The diameter of PCL random fibers was $1.84 \pm 0.14 \mu\text{m}$. From figures 1(c)–(d), the self-assembly of nanofibers into a honeycomb pattern was observed in the scaffolds. The honeycomb pattern was well-organized and distributed uniformly in the electrospun scaffolds, which mimics the tube formation of HUVECs (figure 1(e)–(f)). The average diameter and area of honeycombs were $511 \pm 94 \mu\text{m}$ and $1.33 \pm 0.46 \times 10^5 \mu\text{m}^2$, respectively (see the distribution of diameter and area of honeycomb patterns in figures 2(a) and (b)). Due to the competitive actions of surface tension and electrostatic repulsion, the charged nanofibers could self-assemble into the honeycomb structures, which might explain how the patterns were formed [43, 45]. The wall of honeycomb scaffolds were observed by SEM images with higher magnification (figure S1). Most of the fibers were aligned along the wall of the honeycombs, whereas only few fibers were stretched and suspended in the center of the honeycombs. Moreover, the junctions of the honeycomb patterns tend to form three-branchse with an angle approaching 120° (figures S1(a)–(b)), which is consistent with other electrospun honeycomb studies [43, 44]. Figures S1(c)–(d) clearly showed the beads structure on the wall and the alignment of nanofibers produced by self-organization of the honeycombs. The level of alignment was measured by the coherency coefficients (figure S1(e)). The coherency coefficient

ranges from 0 to 1, with a value of coherency close to 1 indicating a strongly coherent orientation and greater alignment [59]. In order to compare the alignments of nanofibers and beads in different positions of the honeycomb scaffolds, we measured three sections in the honeycomb scaffolds: nanofibers on the wall, beads structure on the wall, and nanofibers in the center of the honeycomb scaffolds. Analysis results demonstrated that the coherency of nanofibers near the wall of the honeycomb was significantly higher (0.81 ± 0.03 vs. 0.23 ± 0.14 , 0.20 ± 0.08 and 0.21 ± 0.05) than that on the beads, center of the honeycomb and the random scaffolds. The fiber randomness at the center of the honeycomb is similar to beads and the random scaffolds. Although the beads structure showed randomly oriented morphology, which could interfere with the coherency measurements of the nanofibers on the wall, the nanofibers next to the beads still showed higher coherency than the central area of the honeycomb and random scaffolds.

The 3D image and depth of the honeycomb structure was obtained by optical profilometry, as shown in figures 2(c)–(e). A complete honeycomb was observed in 3D, which showed a similar pattern as what observed by SEM. It can be seen that the wall of honeycombs has a higher depth compared to the central region. The average depth of honeycomb pattern is approximately $82.7 \pm 26.8 \mu\text{m}$, whereas a lower depth of random scaffolds (less than $2 \mu\text{m}$) was observed in figure S2. These results showed that the walls of the honeycomb nanofibrous scaffolds display a significant height, providing a 3D surface for cell growth as compared to random scaffolds.

3.2. HUVECs on random and honeycomb electrospun scaffolds

Live/dead assays were carried out on random and honeycomb scaffolds after one day of *in vitro* cell culture. The results can be seen in figure S3. After one day of culture, cells attached to and survived very well on both scaffolds (green indicates live cells). Only

Figure 4. Fluorescence images stained for CD31 (a)–(b), VE-Cadherin(c)–(d) and nuclei (DAPI: blue) of HUVECs on the random (a) and (c) and honeycomb (b) and (d) fibrous scaffolds after 5 d culture (scale bar: 100 μ m).

few dead cells were seen on the scaffolds (red indicates dead cells). The percentage of living cells on random and honeycomb scaffolds were $93 \pm 3\%$ and $95 \pm 2\%$, respectively, which indicated good biocompatibility for both scaffolds. Cell viability was evaluated by using the PrestoBlue assay at 1, 3 and 5 d as illustrated in figure 3(a). Results showed that cell viability for both groups gradually increased over 5 d, whereas no significant differences were identified between random and honeycomb scaffolds for the same time point. These observations supported the live/dead findings and confirmed low cytotoxicity of both random and honeycomb nanofibrous scaffolds. In addition, cell proliferation was determined using a CyQUANT™ Cell Proliferation Assay Kit to measure total DNA content. The samples with HUVECs were harvested after 1, 3 and 5 d of culture. The DNA content on honeycomb scaffolds was higher than that on random scaffolds at day 5 (figure 3(b)). There was a statistically significant increase of proliferation on honeycomb scaffolds compared to the random ones. Cell proliferation was also evaluated by Ki67 staining, as it is a marker for proliferation. As shown in figure S4, at day 5, the intensity of Ki67 fluorescence-positive cells on the honeycomb scaffolds was much higher than that on the random scaffolds. These results suggested that honeycomb

patterns in electrospun scaffolds were capable of positively influencing HUVECs growth, ultimately leading to a higher number of cells compared to the random scaffolds. This might be attributed to the 3D structure of honeycomb patterns; indeed, the honeycomb scaffolds have higher depth compared to random scaffolds, which offered increased available surface and large porosity for cell growth.

3.3. Formation and orientation of tube-like structures

With the purpose of investigating the responses of endothelial cells to honeycomb structures, cells cultured on random and honeycomb patterns were observed by immunocytochemistry staining and SEM. Immunocytochemistry staining with CD31, VE-Cadherin and DAPI was used to evaluate cell identity and morphology on the scaffolds. As shown in figure 4, HUVECs cultured on both scaffolds expressed an endothelial cell phenotype showing positive staining of CD31 and VE-Cadherin. However, the cells responded differently to random and honeycomb nanofibrous scaffolds. Cells on random scaffolds were well spread and showed a disorganized distribution without any tube formation, as shown in CD31 staining (figure 4(a)). Electrospun fibers typically form 2D membranes with small pore size and

Figure 5. (a)–(b) 3D confocal images of the HUVECs grow on honeycomb scaffolds showing the capability of cells to generate 3D tube-like structures. (c)–(d) The lumen cavity appears as a negatively stained central space when viewed in horizontal (XY) and vertical (YZ) cross section. Nuclei were labeled with DAPI (blue) and HUVECs were stained with an anti-CD31 antibody (green). Scale bars are 100 μm (a), 20 μm (b), 10 μm (c) and 5 μm (d).

low thickness [60], which typically do not allow cell infiltration and reorganization.

Many researchers have reported that HUVECs grown on random electrospun fibers display a well-spread polygonal morphology and irregular distribution, which should be guided by the densely packed and randomly oriented nanofibers [61–63]. Sun *et al* reported that HUVECs on electrospun cross-linked gelatin membranes formed spherical or spindle morphology with plenty of pseudopodia attached to the fibers [62]. In another study, Li *et al* showed that HUVECs grown on polyurethane (PU) fibers displayed a small spherical or narrow shape and non-spreading morphology [61]. Recent findings explained that changing the mechanical properties (e.g. stiffness) of electrospun fibers could affect cell morphology and the vessel formation capacity of HUVECs [63, 64]. For example, low fiber stiffness of electrospun scaffolds could exhibit sufficiently stretched and expanding cell morphologies, whereas small cell areas and spindle cell morphology were

present for HUVECs grown on stiff electrospun scaffolds. Low fiber stiffness also proved the cell capacity to actively recruit nearby fibers, which increased cell adhesion, infiltration and finally promoted cell migration [65]. In our study, PCL random fibers are most likely stiff for cells, which resulted in no observed penetration or migration into the scaffolds (figure 4(a)).

In contrast, cells on the honeycomb scaffolds showed a preferential localization along the walls of the honeycomb pattern, which is mainly due to the alignment of the nanofibers in the honeycomb scaffolds, and were capable of migrating through the scaffolds thickness (figure S6). It has been widely reported that aligned nanofibers could provide contact guidance cues for endothelial cells [66–68]. In addition, the beads structure on the wall could afford a large attaching area for cells to anchor, which might be another reason to explain the preferential location of HUVECs after seeding. Cord-like structures organized by HUVECs were also formed when cells were

Figure 6. SEM images of HUVECs on random (a) and (b) and honeycomb (c) and (d) PCL fibers after 5 d culture. Scale bars are 50 μm (a) and (c) and 10 μm (b) and (d).

cultured on honeycomb scaffolds (figure 4(b)). This phenomenon resulted in the formation of 3D tube-like structures on honeycomb scaffolds, as also confirmed by 3D confocal images (figure 5). Confocal images of horizontal and vertical (figures 5(c)–(d)) cross sections confirmed the existence of the central lumen, which appeared as a negatively stained central space extending along multiple cell lengths. Figure S5 also illustrated another negatively labeled lumen and the position of this lumen. We quantified about 30 tubes from multiple CD31 staining images on one scaffold and the length of tubes was $247.6 \pm 152.5 \mu\text{m}$. Cell infiltration was analyzed by slicing along the Z-stack obtained from confocal microscopy, from the bottom to top of the honeycomb scaffolds, allowing us to observe cell penetration into the honeycomb pattern (figure S6). These results clearly demonstrate the successful penetration of HUVECs up to 50 μm in the honeycomb scaffolds. HUVECs were also seeded on a 3D printed honeycomb scaffolds in order to supply an additional pattern to assess the specificity of tubular formation. HUVECs grew along the 3D printed scaffolds without showing any clear tubular structure (figure S7). The junction of HUVECs in the scaffolds was further revealed by VE-Cadherin staining (figures 4(c) and (d)). VE-Cadherin is the basic adhesion junction in endothelial cells, which is important for cell to cell contacts [69]. It was interesting to note that more VE-Cadherin (green) was observed when

cells were cultured on the honeycomb nanofibrous scaffolds compared to random scaffolds, indicating that cells generated stronger cohesion and organization of cell-cell junctions. Moreover, the honeycomb pattern on electrospun scaffolds could lead to the ability of HUVECs to contact and form a tube-like structure. As proven previously, the formation of cell-cell connections is essential for endothelial cells to maintain cell-cell adhesion and communication, which subsequently directs endothelial cell morphogenesis and guides the early development of vascular networks [70]. The addition of micro-/nanotextured topographies to materials could effectively activate angiogenic signaling pathways, which are crucial in the process of angiogenesis [71, 72]. A regular pattern such as the honeycomb guarantees that cells in the entire construct are never further away from the next vessel than half of the long diagonal of a honeycomb cell. The results demonstrated in our study therefore supported that the geometries of the honeycomb patterns might also activate angiogenic signaling pathways in HUVECs and stimulate reorganization of cell bodies into tube-like structures.

The morphology of cells grown on both scaffolds was observed by SEM (figure 6). HUVECs cultured on random scaffolds were widely spread and showed polygonal shape without a preferential distribution, whereas those on honeycomb scaffolds grew along the wall of the honeycomb and preferred to retain at

Figure 7. Fluorescence images stained for F-actin (phalloidin: red) and nuclei (DAPI: blue) of hMSCs-HUVECs on random (a) and (c) and honeycomb (b) and (d) PCL electrospun scaffolds after 5 d of co-culture. Scale bars are 100 μm .

the wall. This result is consistent with immunostaining. More SEM images of HUVECs on honeycomb scaffolds with low magnification are shown in figure S8. We also looked into single cells grown on scaffolds (figures 6(b) and (d)). We observed that cells on the random scaffolds were more elliptical with less pseudopodia. In contrast, cells on honeycomb scaffolds were more stretched out, and some pseudopodia were elongated along the fiber direction. More pseudopodia of cells on honeycomb could firmly attach to the nanofibers and might help cells migrate in three dimensions. Interestingly, with smaller fiber diameter in honeycomb scaffolds the pseudopodia were longer and smaller on attached fibers. This observation has also been reported by Li *et al* [73].

3.4. Co-cultures on honeycomb nanofibrous scaffolds

While ECs are the cell lines capable of forming blood vessels in human tissue, perivascular cells, especially pericytes, have been shown to have great impact on vascularization. It was previously demonstrated the hMSCs can act as pericytes and stabilize the formed vascular structures when co-cultured with ECs in defined conditions. Therefore, co-culturing hMSCs with endothelial cells can be an effective strategy

to form vascularization, and to stabilize blood vessels. To determine the topographical influence of honeycomb scaffolds on co-cultured cell behavior, HUVECs and hMSCs were co-cultured up to 5 d. DAPI (blue)/phalloidin (red) staining was performed to observe overall cell distribution and organization.

As shown in figure 7, the honeycomb structure in electrospun scaffolds could guide cell distribution. On random scaffolds, cells exhibited wide spreading of cellular actin filaments and were randomly distributed throughout the nanofibers without any preference. While on honeycomb scaffolds, most of cells distributed on the wall of the honeycomb and stretched along the attached nanofibers. It seems that the cells preferred to spread at the top of the honeycomb pattern, growing along the wall. 3D confocal images also proved this observation (figure S9). The co-culture further corroborated the trend observed in HUVECs monoculture showing that cell behavior can be regulated and guided by honeycomb nanofibrous scaffolds. It was also interesting to note that VE-Cadherin staining was much higher for the HUVECs co-culture with hMSCs on honeycomb scaffolds compared on the random scaffolds, as shown in figure 8. The results demonstrated that HUVECs/hMSCs co-culture on the honeycomb scaffolds generated strong cohesion and organization of

Figure 8. Immunofluorescence staining of VE-Cadherin in HUVECs after co-culture with hMSCs on random (a) and (c) and honeycomb (b) and (d) scaffolds at day 5. Scale bars are 100 μm .

the inter-cellular junctions. However, it was much less on the random scaffolds. This result obtained in the co-culture investigation also supported that the honeycomb scaffolds could facilitate desirable intra-cellular connection and the following endothelial cell morphogenesis.

In HUVECs monocultures, immunofluorescent images showed the relative expression of CD31 and VE-Cadherin on different scaffolds (figure 5). VE-Cadherin expression of HUVECs became more pronounced and cells were observed forming tube-like structure on honeycomb scaffolds after 5 d culture. In hMSCs/HUVECs cocultures, VE-cadherin expression was also relatively higher on honeycomb scaffolds compared to random scaffolds. Especially, cocultures of hMSCs with HUVECs were observed forming obvious network and clear cell-junctions on honeycomb scaffolds compared to HUVECs monoculture (figure S10). hMSCs were located at the surrounding of HUVECs (figure S10(b)), which could be hypothesized to act as pericytes and stabilize the formed vascular structures. The cellular crosstalk between endothelial cells and hMSCs has been well studied [74]. It has been reported that hMSCs can secrete cytokines and angiogenic growth factors, including VEGF [75], Ang-1 [76], IGF-1 [77], and PDGF [78] among others, to influence endothelial cell behavior. In addition, the extracellular matrix produced by hMSCs may support the tube formation

of HUVECs and stabilize vessel networks when the cells are co-cultured in direct contact [79, 80]. Our coculture results also proved that hMSCs facilitated vessel network formation of HUVECs on honeycomb scaffolds.

Finally, it is known that different scaffolds' pattern have a different effect on cell behavior. In this study, we only compared honeycomb scaffolds with random scaffolds, but did not investigate the different size of honeycomb nanofibrous scaffolds or other patterns on guiding endothelial cell organization into tube-like formation. Our previous findings proved that honeycomb nanofibrous scaffolds with different size and gradients could be successfully fabricated with the same electrospinning method, by simply changing electrospun parameters. A more in-depth analysis of different honeycomb scaffolds on endothelial cell morphogenesis should be studied in future research, to further understand how much control over the process of angiogenesis can be provided by such geometric cues. Moreover, future studies should also aim at understanding if honeycomb nanofibrous scaffolds could provide geometric cues able to influence the organization of other types of cells. Understanding whether other types of vascular cells could be guided by honeycomb patterns and form tubular network would be important for the fabrication of vascularized tissues, which has great potential applications in tissue regeneration.

4. Conclusion

In this study, we fabricated honeycomb nanofibrous scaffolds by the self-assembly process of electrospinning. The fabricated honeycomb scaffolds can support endothelial cells adhesion, proliferation, and guide cell migration on the scaffolds. Compared with random electrospun scaffolds, the honeycomb scaffolds significantly promoted HUVECs proliferation and influenced the distribution of cells. It is interesting that the honeycomb electrospun scaffolds can regulate HUVECs morphogenesis into capillary-like structures with a central lumen. Moreover, higher VE-Cadherin staining was observed when HUVECs were mono-cultured and co-cultured with hMSCs on the honeycomb nanofibrous scaffolds compared to random scaffolds. This study provides evidence that endothelial cells are highly sensitive to their local microenvironment and can respond to geometric cues to form capillary networks. These results show that the better geometric control of endothelial cells during angiogenesis give honeycomb scaffolds potential for vascular tissue engineering.

Acknowledgments

T Yao are supported by China Scholarship Council (Grant No. 201508610081). This research project has been made possible thanks to the support of the Dutch Province of Limburg.

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