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Elasticity of fiber meshes from multiblock copolymers influences endothelial cell behavior

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Key words: tensile E-modulus, HUVECs, electrospun fiber meshes, multiblock copolymers

Abstract:

BACKGROUND: The behavior of endothelial cells is remarkably influenced by the physical and biochemical signals from their surrounding microenvironments.

OBJECTIVE: Here, the elasticity of fiber meshes was studied as a design parameter of substrates for endothelial cells in order to modulate angiogenesis.

METHODS: Human umbilical vein endothelial cells (HUVECs) were cultured on electrospun fiber meshes made from polyetheresterurethane (PEEU), differing in their elasticity. Cell morphology, proliferation, migration and angiogenesis of endothelial cells on the degradable substrate meshes were characterized.

RESULTS: The aspect ratio of HUVECs cultured on the fiber meshes from PEEU materials increased with increasing stiffness of the materials. HUVECs cultured on fiber meshes with high stiffness (Young's modulus $E = 4.5 \pm 0.8$ MPa) presented a higher proliferation rate and significantly faster migration velocity, as well as higher tube formation capability than the cells cultured on fiber meshes with low stiffness ($E = 2.6 \pm 0.8$ MPa).

CONCLUSIONS: These results suggested that tuning the fiber meshes' elasticity might be a potential strategy for modulating the formation or regeneration of blood vessels.

1. Introduction

The behavior and functions of cells are modulated by their extracellular environments [1-4]. The extracellular matrix (ECM) not only provides physical support for cells to grow, but also influences cellular behavior through its structural function. The mechanical properties of ECM, such as elasticity, are recognized as an important factor in modulating cell behavior. Cells sense mechanical signals from the ECM, apply force on ECM and further alter ECM composition accordingly [5-8]. The endothelial cell (EC) senses and responds to diverse biochemical signals released by ECs itself or other blood cells in a very complex process [9]. Micro-environmental conditions like temperature, ischemia, hyperosmolality or hyperglycemia have an influence on the behavior of ECs [10]. The cholesterol-enrichment [11] and the increased calcium content [12] significantly increase the stiffness of the vessel wall [13]. The mutual interactions between cells and ECM are the key factors in modulating cells' behavior such as, morphology, growth [14], migration and differentiation [15].

Endothelial cells (ECs) are typically found as a monolayer covering the blood vessel, where they are constantly exposed to various mechanical cues including shear stress and elasticity of the vessel. ECs modulate actin cytoskeleton organization in an integrin-dependent manner and further translate the mechano-stimuli into more meaningful cellular functions including angiogenesis [16-20] in order to respond to mechanical cues. Understanding how the relevant physical cues regulate the angiogenesis process is vital in controlling a variety of favorable cellular events towards regeneration [21,22]. In addition, elucidating the physical cues, which affect angiogenesis, will also aid in the development of optimal biological constructs to restore normal EC functions of injured tissues or organs and to prevent pathological angiogenesis such as in the progress of tumor formation [23]. Knowing these effects, the question is raised as to whether the stiffness of the ECM matrix has an influence on the EC's angiogenesis potential.

An ideal scaffold, mimicking near-physiological environment, provides physical stability with biophysical cues for modulating cell proliferation, developing tissues, and influences stem cell differentiation toward preferred lineages [24]. However, there is a lack of decent experimental systems that mimic the native ECM of the blood vessel, limiting the further discovery of ECs physiological responses to ECM and dissection of the mechanism of ECs force sensing and the downstream signaling networks. Electrospinning has emerged as a promising approach to mimic the native componential and structural aspects of ECM [25-27]. Electrospun fibrous scaffolds provide porous structures with interconnected pore systems, which promote cell recruiting/seeding, adhesion, proliferation, and differentiation.

In this study, an *in vitro* model of the vascular endothelium was designed to investigate the role of EC substrate stiffness on the angiogenesis process. The effect of substrate elasticity on human umbilical vein endothelial cells (HUVECs) behavior was investigated via culturing HUVECs on PEEU electrospun fiber meshes with different elasticities. The cell morphology was carefully studied. We then probe further into whether these fiber meshes of PEEU materials with different elasticities have an influence on endothelial cell behaviors such as migration, proliferation and angiogenesis. Since cell morphology differs with substrate stiffness, and cells respond to substrate stiffness through the integrin binding and traction force of integrin–cytoskeleton, the stiffer substrate contributes to stronger contraction force. Cell shape and cellular traction force modulate cells' movement and proliferation. In addition, the cells' migration and proliferation play a role in forming tube-like network structures, which represent *in vitro* assessment of HUVECs angiogenesis potential. In this manner, we were able to obtain a comprehensive picture of the relationship between stiffness of fiber meshes and the functions of HUVECs.

2. Materials and methods

2.1 Preparation of fiber meshes from PEEU materials

PEEU(x) is obtained by reacting x% (w/w) oligo(p-dioxanone) (PDDO) diol (100-x)% (w/w) poly(ε-caprolactone) (PCL) diol with an equimolar amount of a diisocyanate. The composition of PEEU was varied between 40% (w/w) and 70% (w/w) PPDO. Four types of PEEU materials namely, PEEU40, PEEU50, PEEU60, and PEEU70 were synthesized as described previously [28,29].

The fiber mesh scaffolds made from PEEU materials were prepared by electrospinning. PEEU is dissolved in hexafluoroisopropanol (HFIP) and filtered through a filter with a pore size of 1μm before electrospinning. The electrospinning was performed inside a custom-made chamber with 20% humidity. The collector used is a rotating drum covered with polypropylene film. The applied voltage for electrospinning was adjusted during the whole process to achieve a stable Taylor cone. For cell cultivation, the obtained fiber meshes were sterilized using 10% (v/v) ethylene oxide, 54 ºC, 65% relative humidity, 1.7 bar, 3 hours of gas exposure time, and 21 hours of aeration phase).

2.2 SEM micrographs of fiber meshes from PEEU materials.

The morphology of the fiber meshes were captured by scanning electron microscopy (SEM) (Phenom G2 pro, L.O.T.–Oriel, Darmstadt, Germany). The obtained SEM images were analyzed by Image J software (version 1.44; National Institutes of Health) [30]. To analyse the average diameter, and the diameter distribution of fibers, 100 individual fibers were measured for each sample [31].

2.3 Mechanical properties of fiber meshes from PEEU materials.

The mechanical properties of the fiber meshes from PEEU materials were characterized by tensile tests at 37 °C in dry state with a Zwick Z1.0 tensile tester (Zwick, Ulm, Germany) equipped with a temperature-controlled thermo-chamber (Eurotherm Regler, Limburg, Germany). Tensile tests were carried out at a constant rate of 5 mm per minute. Mats of 10 mm

width and 40 mm length were cut from specimens. The Young's modulus for the scaffold mechanics (E) was calculated from the stress-strain curve at the linear region between 1% and 5% strain. Each sample was tested five times and the results were averaged.

2.4 Cultivation of HUVECs

HUVECs (Lonza, Walkersville, MD, USA) were seeded on PEEU fiber meshes and cultured in endothelial cell growth medium (EGM^{TM}, Lonza, Walkersville, MD, USA) at 37 °C, in a humid atmosphere containing 5% (v/v) CO_2. Cell culture medium was changed every two days. ECs from passages 5 to 7 were used in all described experiments.

2.5 Cell morphology characterization

To study the cell morphology on the fiber meshes, HUVECs were seeded on fiber meshes from PEEU materials with a density of 5.0×10^4 cells/ cm^2. After 3 days cultivation, cells were fixed with 4% (v/v) paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA) for 20 minutes and then were permeabilized with 0.2 % (v/v) Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) for 15 minutes at room temperature. The cytoskeleton and nuclei were stained with ActinRed™ 555 and Hoechst 33342 (Life Technologies, Darmstadt, Germany) according to the protocol supplied by the manufacturer. The stained samples were washed with PBS and then imaged by a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany). Then cells aspect ratio was analyzed using Image J software (National Institutes of Health, USA).

2.6 HUVEC proliferation

HUVECs were seeded on the fiber meshes from PEEU materials at a density of 1×10^4 cells/ cm^2, the cell proliferation was tested by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the protocol that was supplied by the manufacturer. Briefly, at the selected time points, the old medium was replaced with 300 µl of fresh medium, followed by adding 30 µl of CCK-8 solution. After 2 hours of incubation at 37 ºC, 100 µl of

medium/CCK-8 mixture was transferred from each insert into a transparent 96-well tissue culture plate (TCP), and the absorbance was measured at a wavelength of 450 nm using a microplate reader (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland). A standard curve, which was generated by measuring the absorbance of a series of cells with known cell numbers, was used to calculate the number of cells on the fiber meshes.

2.7 HUVEC Migration

HUVECs were seeded on the different fiber meshes of PEEU materials at a density of 1.0 x 10^4 cells/ cm^2. After 7 days cultivation, the cells were harvested and reseeded on 24 well-TCPs with a cell density of 2.0 x 10^4 cells/ well. After 24 h cultivation, the cells' nuclei were stained by Hoechst 33342 (NucBlue® Live Reagent, Life Technologies, Darmstadt, Germany). Cells were observed and recorded every 10 minutes for 15 h by a time-lapse imaging microscope (IX81 motorized inverted microscope, Olympus, Hamburg, Germany) combined with an online cage incubator providing a humidified atmosphere (37 ºC, 5% (v/v) CO_2). The migration trajectories and velocities were analyzed using Image J software (National Institutes of Health, USA) combined with the software plugins ''manual tracking'' and ''chemotaxis and migration tool'' (ibidi GmbH, Martinsried, Germany).

2.8 Analysis of endothelial cell tube formation

HUVECs were cultured on the different fiber meshes for 7 days, and then the cells were harvested. To assess the tube formation ability of HUVECs, Geltrexs Matrix solution (Life Technologies, Darmstadt, Germany) was used to coat 24 well-TCPs according to the given protocol. Subsequently, harvested HUVECs were seeded on the Geltrexs Matrix solution coated wells at a density of 7 x 10^4 cells/ well and 500 µL cell culture medium was added. After 18 h incubation, the formed tubes were visualized and imaged by phase contrast microscopy (AxioSkop, Carl Zeiss, Jena, Germany). In each well, the images were taken in randomly selected observation fields. The number of the closed tubes was counted and the total length of

the tubes was measured using Image J software (National Institutes of Health, USA) combined with the software plugin “Angiogenesis Analyzer”.

2.9 Statistical analysis

The number of replications for experiments was at least three, as indicated respectively in the figure legend for each assay. Statistical analysis was performed using the two-tailed independent-samples t-test, and a significance level $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard deviation.

3. Results

3.1 SEM micrographs and fiber diameter distribution of fiber meshes from PEEU materials.

Polyetheresterurethane (PEEU) multiblock copolymers composed of oligo(p-dioxanone) (PDDO) and poly(ε-caprolactone) segments, are suitable materials for biomedical application [32]. The chemical structure of PEEU multiblock copolymer is shown in Fig. 1(a).

Surface morphologies of PEEU40, PEEU50, PEEU60, and PEEU70 were determined by SEM, and the representative SEM images for fiber meshes are shown in Fig. 1(c, e, g, and i). The histogram of average fiber diameter distributions achieved from analysis of the fiber meshes SEM images are shown in Fig. 1 (c, e, g, and i). Fiber diameter (mean ± SD) of the fiber meshes from PEEU40, PEEU50, PEEU60 and PEEU70 materials are 1.7 ± 0.5 μm, 1.4 ± 0.6 μm, 1.4 ± 0.5 μm, and 1.7 ± 0.4 μm, respectively. There was no substantial difference in fiber diameter among the different groups.

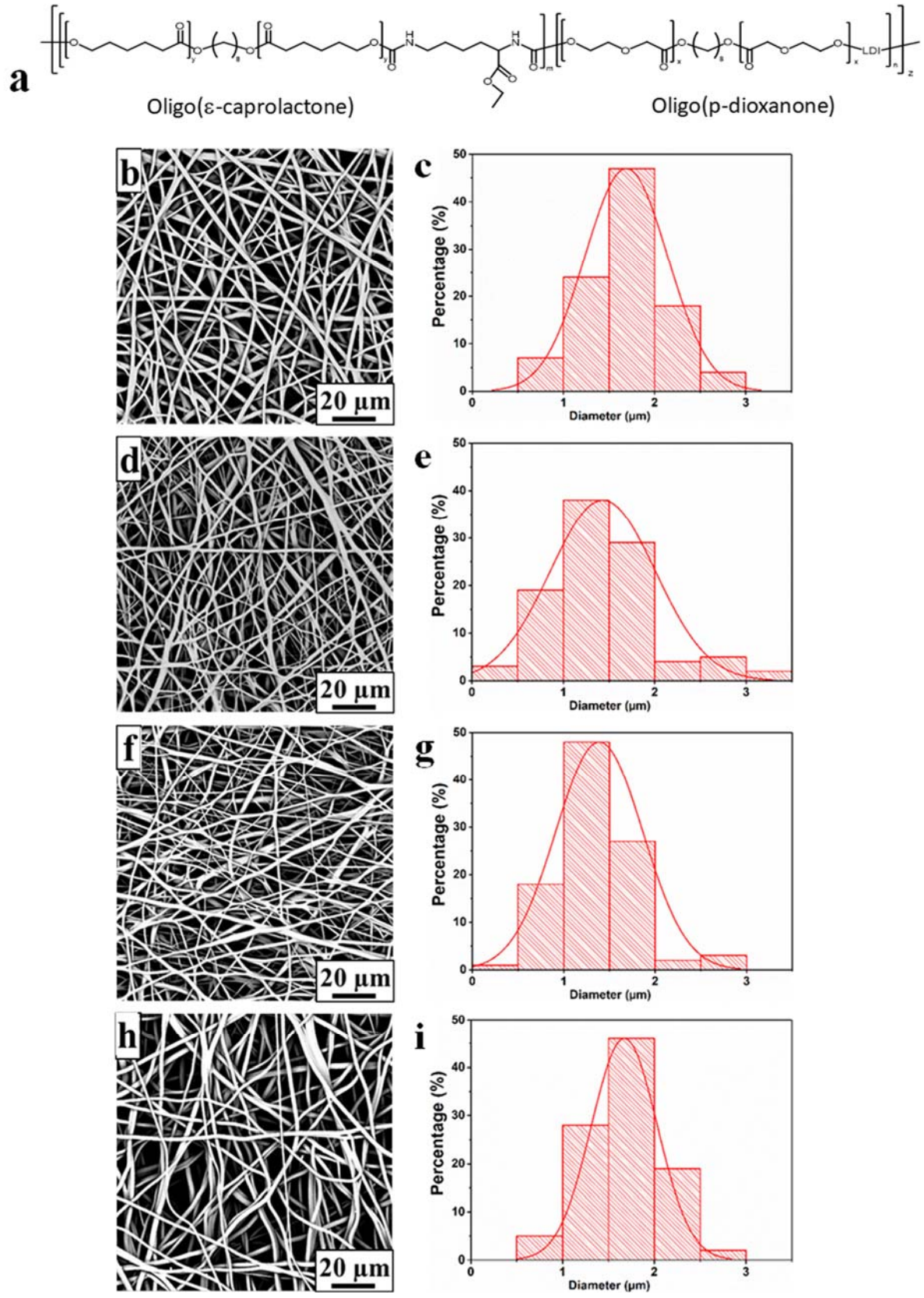

Figure 1. Chemical structure of a PEEU multiblock copolymer (a); SEM images of fiber meshes and histograms of fiber diameter distributions from PEEU40 (b, c), PEEU50 (d, e), PEEU60 f, g), and PEEU70 (h, i) materials.

3.2 Mechanical properties of fiber meshes from PEEU materials.

Tensile tests at 37 °C were performed in dry state in order to examine the mechanical properties of PEEU fiber meshes. The mechanical properties are listed in Table 1. The E-moduli of PEEUs increased from PEEU40 to PEEU70, as the amount of PPDO in the composition was increased. The E-modulus is likely related to the percentage of PPDO in the fibers. For the elongation at break, the trend was related to the molecular weight and associated Van der Waals forces. As measured by GPC, the molecular weights (M_n) of PEEU40 to PEEU70 are shown in Table 1.

Table 1. Mechanical properties of fiber meshes determined by tensile tests in dry state at 37 °C and molecular weight of PEEU materials measured by GPC.

	E-Modulus (Mpa)	Failure stress (Mpa)	Elongation at break (%)	Mesh thickness (μm)	Molecular weight (M_n) (g mol^{-1})
PEEU40	2.6 ± 0.8	1.4 ± 0.4	320 ±110	39.2 ±2.7	47000
PEEU50	3.2 ± 0.9	1.1 ± 0.2	150 ± 15	87.3 ± 10.0	33000
PEEU60	4.0 ± 0.9	1.6 ± 0.1	270 ± 10	77.8 ± 3.9	37000
PEEU70	4.5 ± 0.8	2.8 ± 0.2	410 ± 50	53.8 ± 3.9	61000

3.3 Morphology of endothelial cells

The early spreading and actin polymerization of cells seeded on fiber meshes with different elasticities were evaluated in order to study alterations in expression of cytoskeletal actin. Representative images of HUVECs cultured for 3 days are shown in Fig. 2a. There was a significant drop in the average spreading area of HUVECs, when seeded on PEEU60 and PEEU70. Since actin cytoskeleton provides the driving force for cell spreading, we measured TRITC (Tetramethylrhodamine Isothiocyanate) conjugated phalloidin to label actin filaments (Fig. 2a): It revealed a strong increment of the length of actin filaments of HUVECs. The cells exhibited a flat appearance on fiber meshes of PEEU40 and PEEU50, while, on PEEU60 and PEEU70, cells had cellular elongation and had a higher aspect ratio as shown in Fig. 2b.

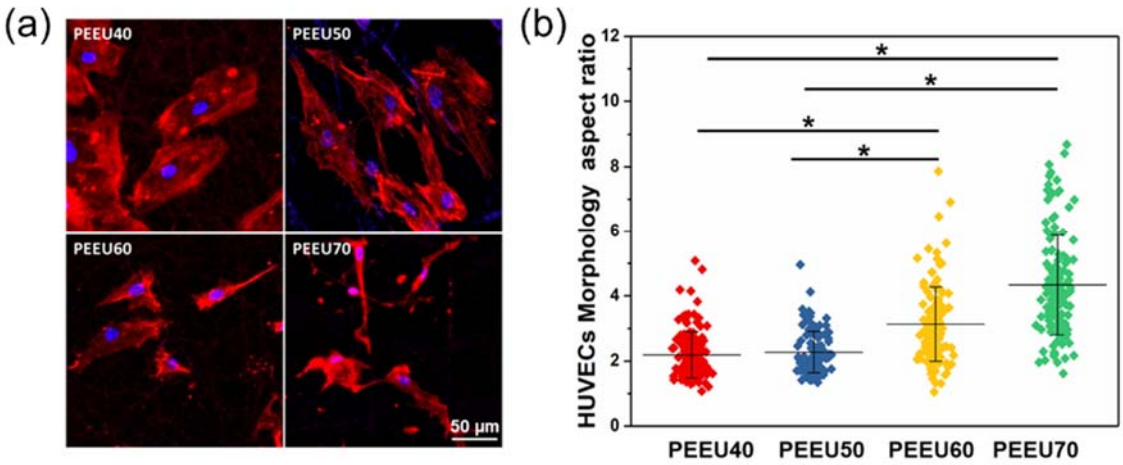

Figure 2. Morphology of HUVECs cultured on fiber meshes from different PEEU materials. (a) HUVECs were stained for Nuclei (blue) and F-actin (red); (b) HUVECs morphology aspect ratio distribution was plotted, (n_{PEEU40} = 238; n_{PEEU50} = 213; n_{PEEU60} = 227; n_{PEEU70} = 239); the data were statistically analyzed by t-test. *$p < 0.05$.

3.4 HUVEC proliferation

In order to test our hypothesis that the elasticity of the fiber meshes plays a role in EC proliferation, the cell growth was compared at different time-points (24-144 hours) after cell seeding (Fig. 3). The initial number of cells (on day 1) attached to fiber meshes of PEEU materials were similar. Cell growth was faster on PEEU fiber meshes with higher elasticity. On day 6, HUVECs cultured on the fiber meshes from PEEU70 showed statistically significantly more cells than cultured on fiber meshes from PEEU40 and PEEU50. HUVECs cultured on the fiber meshes from PEEU60 also showed a higher cell number compared with cells cultured on PEEU40 and PEEU50 but did not show a statistically significant difference among them. PEEU fiber meshes supported HUVEC survival and proliferation. Fiber meshes with a higher elastic modulus accelerated the proliferation of ECs.

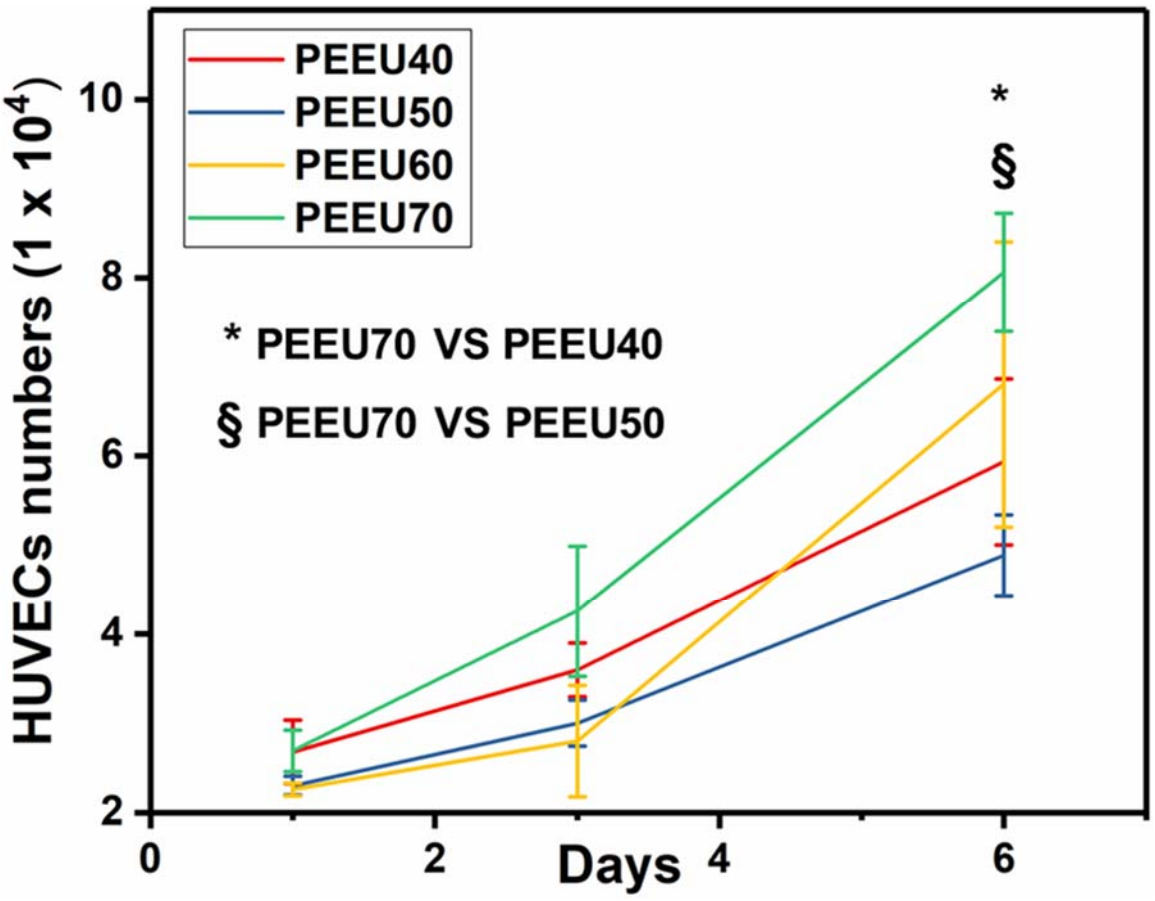

Figure 3. HUVECs proliferation rate cultured on different fiber meshes from PEEU materials. The data were statistically analyzed by *t*-test. (n = 10; * $p < 0.05$ PEEU70 vs PEEU40; § $p < 0.05$ PEEU70 vs PEEU50).

3.5 HUVEC migration

Endothelial cell migration was examined by time-lapse microscopy after cell harvesting from substrates with different elasticities including the migration trajectories and velocities of cells. The single-cell movements are summarized in Fig. 4, which depicts the trajectories of individual endothelial cells, and was obtained by frame-by-frame analyses of the positions of cell-nuclei. The migration velocity of cells harvested from fiber meshes of PEEU40 materials and cells harvested from fiber meshes of PEEU70 materials were 0.058 ± 0.019 (n = 74) and 0.076 ± 0.018 μm/min (n = 54), respectively, the difference of which was found to be statistically significant ($p < 0.05$). Overall, the results from time-lapse imaging experiments clearly demonstrate that elasticity plays an important role in EC migration.

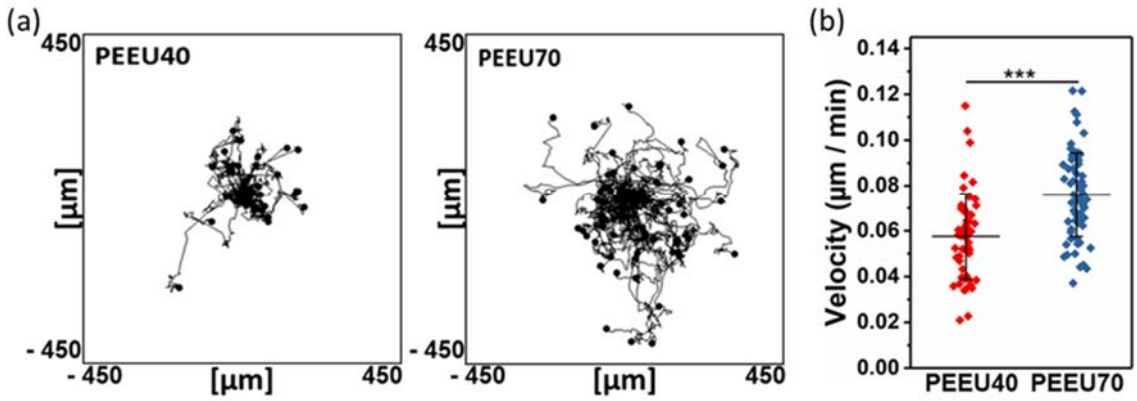

Figure 4. HUVECs migration data of cells cultured on fiber meshes from PEEU40 and PEEU70 materials. (a).The migration tracks of the cells were recorded using a time-lapse microscope and analyzed using Image J. (b) The HUVECs velocity was plotted, ($n_{PEEU40} = 54$; $n_{PEEU70} = 74$); The data were statistically analyzed by t-test. *** $p < 0.001$.

3.6 Analysis of endothelial cell tube formation

The tube formation assay of HUVECs was performed for PEEU40 and PEEU70 fiber meshes, in order to investigate how the elasticity of the fiber meshes of PEEU materials affects HUVEC angiogenesis. Representative images of a tube formation assay with capillary-like tube formation are shown in Fig. 5. Prominent capillary-like networks with a higher number of branching points and longer capillary tube lengths were found for HUVECs from PEEU70. HUVECs harvested from the fiber meshes made from PEEU70 formed an increased number of closed loops (around 13.6 ± 5.6 per square millimeter) while HUVECs harvested from fiber meshes of PEEU40 formed 7.3 ± 2.4 completely closed tubes per square millimeter. The total tube length was around 10000 ± 1500 µm and 11500 ± 1000 µm per field of view of cells harvested from fiber meshes of PEEU40 and PEEU70. Our results indicated that PEEU70 substrate possesses a greater proangiogenic potential for inducing stronger endothelial cell differentiation.

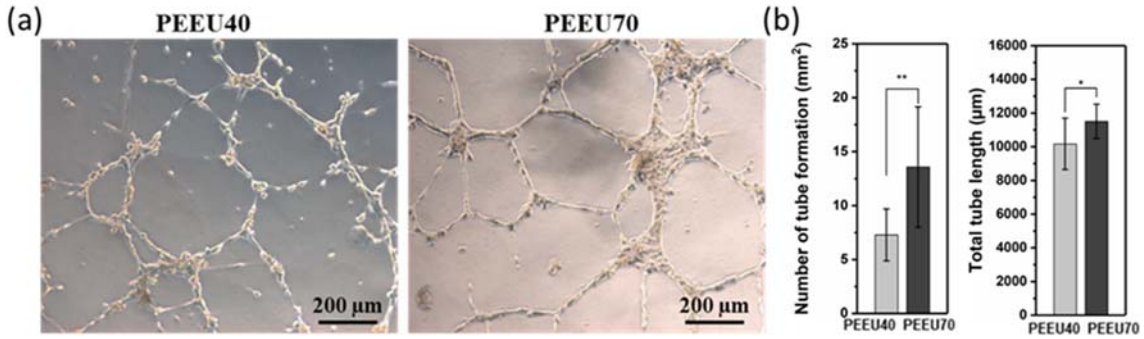

Figure 5. HUVECs tube formation assay. (a) Representative images showed the tube formation of HUVECs from cells cultured on fiber meshes PEEU40 and PEEU70, respectively. (b) The number of the closed tubes were counted and total tube length was measured in captured images ($n_{PEEU40} = 9$; $n_{PEEU70} = 10$); The data were statistically analyzed by t-test. * $p < 0.05$; ** $p < 0.01$.

Discussion

The physical cues from electrospun fiber meshes influence cell behavior remarkably. It has been reported that cell proliferation and morphology were affected by the fiber diameter and orientation [33]. The PEEU materials contain segments from degradable polymers, which are established in clinical applications such as surgical sutures. The segments are connected through a diurethane junction unit, which is chemically based on *L*-lysine. Previous studies showed that PEEU materials were capable to enhance the adhesion of endothelial cells and showed no significant negative influence on vitality and cell cycle behavior [28]. For this purpose, it is important to know whether the fiber elasticity could influence endothelial cell behavior. These fiber meshes had a similarly high porosity and the average diameter of the fibers is 1.5 ± 0.5 μm, which presents a high surface to volume ratio that maximizes cell−material contact area and can be beneficial for cell attachment and growth.

HUVECs are cells derived from the endothelium of veins from the umbilical cord, which provide an *in vitro* model for studying the function and pathology of endothelial cells (e.g., angiogenesis) [20]. HUVECs can perceive the physical cues of the local environment such as elasticity and change their cellular behavior in response. In this study, we found that HUVECs

presented good compatibility for cell adhesion on all fiber meshes, while showing different cell morphologies. HUVECs cultured on the higher E-modulus substrate, PEEU70 fiber mesh, presented a higher cell aspect ratio than that of cells on softer fiber mesh (PEEU40 and PEEU50). The result is consistent with previous reports, in which HUVECs showed round shapes on soft substrates compared to more elongated spread-out shapes on hard substrates [34]. In addition to regulating HUVECs morphologies, the elasticity of fiber mesh also regulates the proliferation of HUVECs. HUVECs presented higher proliferation on the PEEU70 fiber meshes than on PEEU40 and PEEU50 fiber meshes, indicating that the fiber meshes with a higher E-modulus could enhance HUVECs proliferation. This result is consistent with the results of a previously published article, in which it was demonstrated that the stiffness of a substrate influences endothelial cell proliferation via regulating the activation of the SEPT9/Src/Vav2/RhoA pathway [14]. In addition, highly stiff substrates can induce YAP/TAZ nuclear translocalization and then activate the expression of multiple downstream target genes, and subsequently promote cell proliferation, migration, and angiogenesis [35-37]. Our results also showed that cells cultured on fiber meshes with higher E-modulus (i.e. made from PEEU70 materials) had a faster migration velocity and promoted angiogenesis than cells cultured on fiber meshes from PEEU40 materials with a lower E-modulus. This phenomenon also was described in previous studies [37] and can be explained as cells sense and respond to the elasticity of the substrate by the integrin binding and traction force of integrin–cytoskeleton linkages [38], the stiffer substrate contributes to stronger contraction force which induces movement of the cells. The enhancement of endothelial cell proliferation and migration improves the ability of cells to self-assemble into tube-like network structures [39]. The tube formation results showed that cells harvested from PEEU70 fiber meshes formed more closed tubes, and had a longer total tube length than those harvested from PEEU 40 fiber meshes. These results indicated that cells cultured on the stiffer substrate possess a greater proangiogenic potential.

Overall, HUVECs sense and response to fiber meshes made from PEEU materials containing different elasticity properties. The cells on higher stiffness fiber meshes (PEEU70) presented elongated morphologies and enhanced cell proliferation and migration, as well as promoted angiogenesis ability than that on softer fiber meshes (PEEU40).

4. Conclusion

This study supports the notion that endothelial cells are sensitive to the elasticity of fiber mesh substrates. The fiber meshes made from PEEU materials with suitable mechanical properties could improve the angiogenesis potential of endothelial cells. This insight, which physical cues influence endothelial cell behaviors provides meaningful design criteria for biomimetic fibrous scaffolds, could improve vascular regeneration in the future.

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