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Effects of aligned electrospun fibers with different diameters on hemocompatibility, cell behaviors and inflammation in vitro

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Abstract

An endothelial cell (EC) monolayer aligned along the direction of blood flow in vivo shows excellent capacity for anti-inflammation and anti-thrombosis. Therefore, aligned electrospun fibers have been much studied in the field of vascular implants since they are considered to facilitate the formation of an aligned EC monolayer, yet few research studies have been comprehensively reported concerning the effects of diameter scales of aligned fibers. In the present work, a series of aligned polycaprolactone (PCL) electrospun fibers with varying diameters ranging from dozens of nanometers to several micrometers were developed, and the effects of the fiber scales on EC behaviors, hemocompatibility as well as inflammatory cell behaviors were investigated, to evaluate their potential performance in the field of vascular implants. Our results showed that platelets exhibited small attachment forces on all fibers, and the anticoagulation property improved with the decrease of the fiber diameters. The impact of fiber diameters on human umbilical vein endothelial cell (HUVEC) adhesion and NO release was limited, while significant on HUVEC proliferation. With the increase of the fiber diameters, the elongation of HUVECs on our samples increased first then decreased, and exhibited maximum elongation degrees on 2738 nm and 2036 nm due to the strong contact guidance effect on these graphical cues; too thick or too fine fibers would weaken the contact guidance effect. Furthermore, we hypothesized that HUVECs cultured on 2036 nm had the smallest spreading area because of their elongation, but 2738 nm restricted HUVECs spreading limitedly. Similarly, NO production of HUVECs showed a similar change trend as their elongation degrees on different fibers. Except for 2036 nm, it exhibited the second highest NO production. For RAW 264.7 cells, poorer cell adhesion and lower TNF-α concentration of 1456 nm indicated its superior anti-inflammation property, while 73 nm showed a contrasting performance. Overall, these findings partly revealed the relationship between different topographies and cell behaviors, providing basic insight into vascular implant design.

1. Introduction

Vascular transplantation is the most effective means of treating cardiovascular diseases, while the limited availability of healthy and mechanically robust autologous, allogeneic, or xenogeneic vein sources has stimulated the development of artificial substitutes [1, 2]. Dacron and ePTFE have been successfully utilized for large-diameter (inner diameter >6 mm) artificial vessel replacements. However, because of the disappointing long-term patency that is caused by the intimal hyperplasia and high risk of luminal thrombosis, there is no a successful commercial small-diameter vessel graft up to now [3, 4]. The innermost layer of native blood vessels is the endothelium, which consists of a continuous monolayer of endothelial cells and directly contacts with blood. The endothelium is involved in multi-faceted blood vessel activities, such
as inflammation, fibrinolysis, hemostasis and extra-
cellular matrix (ECM) production [1, 5, 6]. Import-
antly, the endothelium could act as an anti-
coagulating barrier to avoid thrombus formation by
inhibiting platelet adhesion. Unfortunately, artificial
vascular grafts cannot spontaneously realize endothe-
lialization in situ due to low endothelial cell initial
attachment, cell spreading and growth [7–9]. There-
fore, enhancing the endothelialization of artificial
vascular grafts has become a promising research
direction of vascular tissue engineering development.

In vivo, endothelial cells (ECs) attach on basement
membranes (BMs), Collagen IV, laminins, nidogens,
elastin and other functional components of BMs orga-
nize into or associate with BMs to provide vital physical
support for the vascular endothelium, and also provide
some individual signals to cells and tissues [10–12]. The native BMs exhibit a complex meshwork
topography with nanoscale range (1–100 nm) fibers and submicron range (100–1000 nm) pores [13]. ECs
exhibit an elongated morphology aligned along the
direction of blood flow because of the flow-mediated
mechanotransduction effects, which could lead to
cytoskeletal rearrangement and paralleled stress fiber
bundles [14–16]. In addition to the shear force of
blood flow, the topographic cues of BMs also con-
tribute to the elongated morphology of ECs [17, 18].
The topographic cues of ECs-attached surfaces could
affect the ECs’ activities, including cell adhesion, cell
proliferation, cell orientation and cytoskeleton
arrangement [6, 19–21]. Importantly, research has
revealed that the monolayer of oriented ECs aligned
along the direction of blood flow played crucial roles in regulating anti-thrombogenic in the blood
vessel walls and also in pro-inflammatory reactions
[18, 21–24]. Besides, an aligned morphology with the
direction of blood flow allows the endothelial layer a
high shear stress resistance and lowest flow distur-
bance [25, 26]. Thus, many researchers have been
attempting to mimic the aligned structure of ECs on
the inner surfaces of vascular implants. The grooved
surfaces and the aligned electrospun fibers have always
been reported to induce the alignment of cells.

Studies showed confusing conclusions on the
effects of the structure parameters of grooves, and also
the diameters of electrospun fibers on hemocompat-
ibility and ECs’ activities [1, 18, 22, 26–32]. With
regards to hemocompatibility, Milleret et al found that
small-diameter electrospun fibers, as well as casting
film, could inhibit the adhesion and activation of pla-
telets [33]. Cutiongco et al also reported the superior
hemocompatibility of unpatterned film compared to
patterned film [34]; However, Ding [35] and Lamich-
hane [36] reached the opposite conclusion. They
found that when compared to smooth substrates, elec-
 trospun or grooved substrates could reduce platelets’
adhesion and activation more. Chen et al used micro-
grooved polydimethylsiloxane substrates with the
same groove width and ridge width of 3, 5, 10 and
30 μm to guide EC orientation [18]. The results
demonstrated that an EC monolayer on smaller-sized
groove substrates exhibited better anti-thrombotic
capability. Besides hemocompatibility, there is also no
clear consensus on the effect of surface topography
(nanofibers, microfibers) on EC behaviors. Li et al
evaluated the HUVEC behaviors on PCL electrospun
substrates with different diameter and orientation
[37]. They found that nano-sized fibers (about 300 nm)
could enhance EC adhesion and proliferation
remarkably, while micro-aligned fibers could induce a
better alignment of ECs than nano-aligned fibers
because of the active size range of the contact guidance
effect. The research of Ko et al strengthened these
views [38]. On the contrary, Marissa and Bryce found
that ECs presented better aligned and elongated
morphology on aligned nanofibers (100 and 300 nm)
and on microfibers (1200 nm) [30]. Furthermore,
according to the study of Xu et al, compared to rough
electrospun surfaces, EC function was improved more
on smooth surfaces [39]. However, Zhang et al repor-
ted that nanofiber substrates could enhance the for-
mation of a functional EC monolayer [40].

In the present study, we sought to explore the
effect of topographies of different aligned electrospun
fibers on hemocompatibility and EC behaviors, aim-
ing to provide insights into the optimal design of vas-
cular implants. Also, macrophage cells were utilized
to evaluate the anti-inflammatory property of different
fiber samples, because inflammation should be con-
idered after implantation of vascular grafts. The
inflammatory reaction usually occurs within a few
days to weeks, which could lead to thrombosis and
other unpredictable complications [41–44]. Here, we
fabricated and characterized six different aligned elec-
 trospun fibers, ranging from dozens of nanometers to
several micrometers, followed by assessment of hemo-
compatibility (anticoagulation assay, platelet interac-
tions), EC behaviors (adhesion and proliferation,
viability and morphology, NO release), and macro-
phase viability as well as tissue necrosis factor-α
(TNF-α) production.

2. Materials and methods

2.1. Materials
Polycaprolactone (PCL, Mn ∼80 kDa) was obtained
from Sigma-Aldrich (St. Louis, MO, USA). Trichlor-
oromethane (CHL), methanol and acetic acid (AA) were
purchased from Lingfeng Chemical Reagent Co., Ltd
(China). Formic acid (FA) and alcohol were supplied
by Sinopharm Chemical Reagent Co., Ltd (China).
Bovine serum albumin (BSA) and BCA Protein Assay
Kits were obtained from Sangon Biotech (China).
Human umbilical vein endothelial cells and RAW
264.7 cells were purchased from the Institute of
Biochemistry and Cell Biology (Chinese Academy of
Sciences, China). Roswell Park Memorial Institute
Table 1. Electrospinning parameters of varying fibers.

| Fibers       | Solvent system  | Concentration (w/v) | Voltage (kV) | Distance (cm) | Speed (ml h⁻¹) |
|--------------|-----------------|---------------------|--------------|---------------|----------------|
| 73 nm        | FA/AA 7:3 v/v    | 10%                 | 20           | 17            | 0.1            |
| 582 nm       | CHL/Methanol 7:3 v/v | 15%                | 15           | 15            | 1              |
| 1456 nm      | CHL/Alcohol 7:3 v/v | 15%               | 22           | 20            | 1              |
| 2036 nm      | CHL/Alcohol 7:3 v/v | 20%               | 16           | 20            | 1              |
| 2738 nm      | CHL/Alcohol 7:3 v/v | 20%               | 20           | 20            | 2              |
| 3442 nm      | CHL/Alcohol 7:3 v/v | 20%               | 20           | 20            | 3              |

(RPMI-1640) (Gibco 31800022), DMEM (Invitrogen 11960-044), fetal bovine serum (Gibco 10437028), penicillin–streptomycin solution (Gibco 15140122), Glutamix (Invitrogen 35050-061) and sodium pyruvate (Invitrogen 11360-070) were purchased from ThermoFisher Scientific (USA). CCK-8, Triton X-100, Rhodamine phalloidin and 40,6-diamidino-2-phenylindole (DAPI) were purchased from Yeasen Biotechnology Co., Ltd (China). A live/dead viability kit (KGAF001) was obtained from KeyGEN BioTECH (China). A nitric oxide assay kit (S0021) and rat TNF-α ELISA kit (PT516) were supplied by Beyotime (China). The water used in this study was purified by a Milli-Q system (Millipore, Billerica, Massachusetts, United States). All the chemicals were of analytical grade and were used without further treatment if not specially mentioned.

2.2. Fabrication of aligned electrospun fibers

Electrospun solutions were prepared by dissolving PCL for 24 h at room temperature in mixed solvents of formic acid and acetic acid (7:3 v/v) at a concentration of 10 wt%, chloroform and methanol (7:3 v/v) at 15 wt%, as well as chloroform and ethanol (7:3 v/v) at 15 wt% and 20 wt%, for the fabrication of nanoscale to microscale fibers, respectively. A roller collector was used to fabricate the aligned fibers and the rotator speed was set at 1500 rpm for all fabrication (10 m s⁻¹). Table 1 shows the detailed electrospinning parameters. All membranes were dried in a vacuum oven for 24 h to remove the residual organic solvent.

A field emission scanning electron microscope (FE-SEM, Hitachi SU8010, Japan) was applied to characterize the morphological properties of different fibers. The diameters of the fibers were measured using Image J software. The contact angle instrument (OCA15EC, Germany) was used to measure the wettability of membranes, and PBS solution rather than water was chosen for the measurement.

2.3. BSA adsorption

BSA adsorption on the different sample fibers was assessed utilizing a simplified method. Briefly, the samples were cut into disks 14 mm in diameter and placed in separate wells of a 24-well culture plate, then sterilized with 75% ethanol solution and rinsed in PBS solution three times. Then, 1 ml of BSA solution (1 mg ml⁻¹) was injected into each well, followed by incubation of the samples in a shaker for 6 h at 37 °C. In the present study, we measured the absorbance of the supernatant to evaluate the absorbed BSA on varying samples by using a BCA protein assay kit, rather than the actual protein adsorption.

2.4. Platelet adhesion

Platelet tests were applied to investigate the anti-thrombogenicity of the samples, also the quantity and morphology of the platelets. Centrifuging the anti-coagulated rabbit blood at 1500 rpm for 15 min at room temperature to obtain platelet-rich-plasma (PRP) firstly, for the platelet adhesion test, samples were incubated in PBS for 1 h at 37 °C. After that, 500 μl PRP was added onto each sample surface cautiously and then incubated at 37 °C for 2 h, followed by rinsing three times with PBS to wash away weakly adherent platelets. The adhered platelets were submerged in 2.5 wt% glutaraldehyde solution (in PBS) at 4 °C for 1 day. Eventually, samples were dehydrated at a series of ethanol solution (50%, 70%, 80%, 90% and 100%) and dried in a vacuum drying oven before imaging with FE-SEM (Hitachi SU8010, Japan) [3, 45].

2.5. Anticoagulation assay

A whole blood kinetic clotting time method was applied to analyze the coagulation rate of different samples. All kinds of samples were punched into round pieces with a 14 mm diameter and placed in 24-well plates individually. Then, 100 μl anticoagulated whole blood obtained from rabbits was dropped onto the surfaces of the samples, adding 20 μl of CaCl₂ solution (0.2 M) into the blood of each well to start the blood coagulation cascade, followed by incubation for 30 and 60 min at 37 °C. After that, 2.5 ml distilled water was injected into each well, then incubated at 37 °C for 5 min. For the control group, we used 100 μl of anticoagulated whole blood mingling with 2.5 ml distilled water with no samples. The addition of distilled water lysed the red blood cells that had not been trapped in thrombi and then free hemoglobin was dispersed in the reacted solution. For clotting time measurement, each well was sampled in triplicate (200 μl each) to determine the relative amounts of free hemoglobin in the reacted solutions by the optical density at 540 nm (OD₅₄₀) with a microplate reader MULTISKAN FC (Thermo scientific, USA). The coagulation index values (BCI) were then determined.
were stained at 6 h after ef

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2.7.1. HUVEC adhesion and proliferation

2.7. Biological characterization

2.6. Cell culture

Before cell seeding, all samples were tailored into round pieces and placed in 24-well plates, then sterilized by 75% (v/v) ethanol, followed by rinsing with PBS three times to remove the ethanol. HUVECs and RAW 264.7 cells were utilized in the present study. HUVECs were seeded onto samples at a density of 4 × 10⁴ cells per well for cell adhesion analysis, 1 × 10⁴ cells per well for cell proliferation and viability analysis, then incubated in RPMI-1640 supplemented with 10% fetal bovine serum, 1% glutamate and 1% sodium pyruvate. For NO production test, the RPMI-1640 was changed to DMEM. The culture medium was exchanged every two days. RAW 264.7 cells were seeded onto samples at a density of 2 × 10⁴ cells per well for evaluation of cell viability and TNF-α production, and grown in a cell culture medium (including 88% DMEM, 10% fetal bovine serum, 1% sodium pyruvate and 1% penicillin–streptomycin solution). All the cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

2.7. Biological characterization

2.7.1. HUVEC adhesion and proliferation

To investigate cell adhesion, the nuclei of HUVECs were stained at 6 h after efficient rinsing to wash away the floating cells. The actual numbers of HUVECs on different samples were counted and transferred to cell adhesion densities for evaluation. A CCK-8 assay was utilized to assess HUVEC at days 1, 3 and 5. The culture medium was removed, and the samples were rinsed gently three times using PBS prior to a CCK-8 assay. Then, 50 μl CCK-8 solution mingled in 450 μl RPMI medium was added per well. After incubation (37 °C, 5% CO₂) for 2 h, 100 μl solution was pipetted into wells of a 96-well plate, triplicate per well. The amount of HUVEC was determined by the optical absorbance values at 450 nm, using a microplate reader MULTISKAN FC (Thermo scientific, USA).

2.7.2. HUVEC viability

The cell viability was determined by the numbers of living and dead cells at day 5, using a live/dead viability kit. First, the medium was removed in the wells, then the samples and HUVECs were washed carefully with PBS, followed by addition of the appropriate amount of staining solution onto the samples according to the instructions. After that, the samples were incubated for 30 min at room temperature prior to the cell observation with a fluorescence microscope (Ti-S, Nikon, Japan). This kit contained green fluorescent Calcein-AM to image the cytoplasm of living cells, and red-fluorescent propidium iodide to view the dead cells by penetrating broken cellular membranes.

2.7.3. HUVEC morphology

F-actin organization and the nucleus of HUVECs were imaged by fluorescent staining. Firstly, cells cultured on the samples were fixed with 4% paraformaldehyde at 37 °C for 30 min, then washed with PBS three times and permeabilized using 0.5% Triton X-100 in PBS for 10 min, followed by incubation in 1% BSA (in PBS) for 20 min to weaken nonspecific background staining, and washed with PBS three times again. The following fluorescent staining process was conducted away from light. For nucleus and cytoskeleton staining, the cells were stained with Rhodamine phalloidin for 30 min at room temperature, then washed with PBS three times again. After that, the cells were stained with 40, 6-diamidino-2-phenylindole (DAPI) at room temperature for 2 min, then rinsed with PBS three times again. Cells were observed using a fluorescence microscope (Ti-S, Nikon, Japan). The morphology characterization of cells was assessed using Image J software. The alignment and elongation of cell nuclei were analyzed according to a previous study [47]. Briefly, the outlines of nuclei were best fitted to ellipses, and the value of the major axis/minor axis was defined as the aspect ratio.

2.7.4. NO production of HUVECs

Healthy endothelium could continuously release NO, which is a predominant product that acts as anti-thrombogenic as well as anti-proliferative agents for smooth muscle cells [48–51]. NO release of ECs could be indicated by the content of nitrite, which is a stable metabolite of NO. After incubation for 2 days, the amount of NO released in the culture media was assessed by using a nitric oxide assay kit. The NO production instead of the specific production values (NO concentration) of all samples were calculated and evaluated in the present study.

2.7.5. Viability and TNF-α production of RAW 264.7 cells

The CCK-8 assay was utilized to assess the viability of a RAW 264.7 cell at 24 h, and the detailed procedure was performed as described in section 2.7.1. After incubation for 24 h, the culture medium was collected, then TNF-α production was assessed by applying a rat TNF-α ELISA kit, according to the manufacturer’s recommended protocol.

2.8. Statistical analysis

All results were presented as means ± standard deviation. A one-way analysis of variance (ANOVA) and LSD test were employed to statistically analyze the significant differences between groups of data with SPSS software (version 16.0). A difference was
considered significant when \( P < 0.05(*) \), \( P < 0.01(**) \) and \( P < 0.001(***) \).

3. Results and discussion

3.1. Morphology of aligned electrospun fibers

In the present study, six aligned fibers with significantly different diameters were fabricated by tuning the solvent system, solution concentration and other electrospinning parameters. A roller collector was used to fabricate the aligned fibers, and the rotator speed was set as 1500 rpm for all fabrication (corresponding to the linear velocity of 10 m s\(^{-1}\)). According to studies on aligned electrospun fibers \([29, 30, 52, 53]\), we believed that the rotator speed was high enough to fabricate aligned fibers, though the flow rates and PCL solution were different. The high-resolution morphologies of different PCL fibers were observed by FE-SEM (figure 1), and all of them exhibited well-aligned topography. Quantitative analysis of the FE-SEM images showed that the fiber diameters in the present study ranged from the nanometer to micrometer, and they are referred to hereafter as 73 nm, 582 nm, 1456 nm, 2036 nm, 2738 nm and 3442 nm, respectively, according to their diameter values. In vivo, endothelial cells adhere on the vascular basement membranes, which consist of submicron (100–1000 nm) pores and nanoscale (1–100 nm) fibers. The fibrillar network of 73 nm was the most similar to the diameter scale of the native vascular basement membranes among all the fibers, though our fiber samples were aligned.

3.2. Wettability and BSA adsorption of aligned electrospun fibers

In the present study, PBS solution as the simulated body fluid was applied to measure the contact angles of samples rather than water. PBS contact angles can reflect the wettability of different samples in the human body more precisely. As shown in figure 2, 2036 nm showed the lowest contact angle of 126.30°, and 582 nm showed the highest contact angle of 132.43°. The PBS contact angles of 2738 nm and 3442 nm were 131.08° and 131.68°, respectively, and there was no difference between them, also with 582 nm. Though there were always some significant differences between the different samples as shown in figure 2 according to the results of the one-way analysis of variance (ANOVA) and LSD test, all samples were hydrophobic, and the difference values were small. The varied topographies of the samples contributed to the changes in the PBS contact angle \([32]\).

The wettability could influence the proteins’ adsorption, which would affect hemocompatibility and the behaviors of the attached cells \([54, 55]\). When biomaterials are implanted into biological environments, protein adsorbed onto the material surfaces is believed to be the first event, then cells interact with the protein layers. Therefore, the adsorbed proteins could affect cell attachment and other behaviors \([56]\). Albumin is a principal constituent of the adsorbed proteins. The adsorption of albumin could avoid platelet activation, while other proteins such as fibrinogen could induce the activation of platelets and a blood coagulation system \([57]\). Thus, higher albumin
adsorption corresponds to preferable blood compatibility. Moreover, research showed that proteins adsorbed on the hydrophobic surfaces via hydrophobic interactions, meaning that hydrophobicity enhances protein adsorption \[58, 59\].

In Figure 3, 73 nm, 582 nm and 1456 nm showed relatively high absorbance, and there was no significant difference between them. We found that 3442 nm had the lowest value, which approximated to 2738 nm, and the value of 2036 nm was a little higher than them. However, no significant difference could be detected among these three samples. In the meantime, there was also no significant difference between the values of 2036 nm with 73 nm, 582 nm and 1456 nm. Based on our experiment protocol, the results revealed that 2738 nm and 3442 nm could lead to more BSA adsorbed than the other four samples. Compared to other samples, 2732 nm and 3442 nm were more hydrophobic, though their larger diameters corresponded to a smaller surface area. The positive effect of hydrophobicity counteracted the adverse effect of the small surface area, leading to more BSA adsorption \[32, 56\]. The contact angle of 582 nm was the highest, but the surface area limited BSA adsorption. We found that 73 nm showed the largest surface area, but the lowest BSA adsorption. We speculated that the reason might be that the PBS contact angle of it reached 129.72°, which is more hydrophilic than that of 2738 nm and 3442 nm. Also, 73 nm exhibited the most similar nano-dimensions with proteins, which might restrict the proteins’ adsorption and affect the conformation of proteins \[60\]. Even though the large surface area and hydrophobicity of materials could lead to an increase in protein adsorption, the conformation of proteins might change, which would further affect cell behaviors. Lord \[61\] and Arima \[54\] confirmed that the conformation of fibronectin adsorbed on the nano-rough surfaces had changed, leading to reduced binding sites for cell adhesion.
3.3. Hemocompatibility of aligned electrospun fibers

Excellent hemocompatibility is a prerequisite for long-time patency of implanted vascular grafts [21, 55, 62]. However, no clear consensus has been reached on the effect of surface topography (nanofibers, microfibers, or smooth surface) on hemocompatibility [55]. Millere et al reported that electrospun grafts with small-diameter fiber and low roughness could inhibit platelets’ adhesion and activation and also prevent coagulation [33]. In contrast, other studies showed that grooved or electrospun substrates reduced the adhesion and activation of platelets when compared with smooth surfaces [35, 36]. Here, we developed different electrospun membranes with varying fiber diameters to investigate their hemocompatibility.

Platelet adhesion was applied to evaluate the effect of different electrospun fibers on the risk of thrombosis. As shown in figure 4, the platelets that adhered on all the samples exhibited round shapes, generally indicating a low attachment force to the samples [1]. The numbers of attached platelets on 73 nm, 582 nm and 1456 nm were 1.13 × 10⁶ platelets/cm², 0.11 × 10⁶ platelets/cm² and 0.32 × 10⁶ platelets/cm², respectively, while the actual platelet numbers on the other three samples were hardly counted accurately because of their large pores sizes. A platelet’s diameter is typically 2–3 µm and is much smaller than the pore size of 2036 nm, 2738 nm and 3442 nm. Therefore, the porous structures could induce platelet retention within the pores individually or collectively, and can result in further detrimental coagulation [2]. Our results showed that the most significant number of platelets adhered onto 73 nm, in agreement with the viewpoint of Kikuchi et al, who demonstrated that substrates with a higher specific surface area were more likely to adhere to platelets [3]. According to MI’s report, the decrease of the water contact angle and increase of surface roughness could both cause increased platelet adhesion [1]. In the present study, the number of platelets on 1456 nm was higher than on 582 nm. Although 582 nm possessed a higher surface area, its lower surface roughness with finer fiber diameter and higher PBS contact angle than 1456 nm led to fewer platelets being adhered. Remarkably, almost no platelets adhered onto 2036 nm. We speculated that the lowest PBS contact angle among all the samples might contribute to it [1], though it might be possible that the platelets had infiltrated into the pores. The topographical cues of 2036 nm also could be another reason for its excellent resistance to platelet adhesion, and intensive and elaborate explorations are required to explain it.

The anticoagulation test was also applied to evaluate the hemocompatibility of varying samples in the present study, and the results were shown in figure 5. Based on the study of Wu et al [63], the coagulation indexes would be high at 5 min compared to all other time points for all samples because of an insufficient mixing of the coagulation-inducing Ca²⁺ ions with the whole blood samples, and the coagulation reaction was completed and saturated after 10 min. Here, we chose two reaction time points, 30 min and 60 min. As shown in figure 5, 73 nm exhibited the highest BCI values among all the samples both at 30 min and 60 min, implying its best anticoagulation property. The BCI values of 582 nm and 1456 nm were approximate with no significant difference between them, and we found that their BCI values were close to that of glass coverslips. Glass coverslips were employed as a positive control since they are not anticoagulant. The coagulation index of the glass coverslip in Wu’s study was 0.054 after 60 min incubation [63]. However, our results showed a significantly higher coagulation index of 0.1819 at 60 min (figure 5). We speculated that the differences in the blood, glass coverslips, and experimental operations might lead to the discrepant coagulation indexes. The glass coverslip should always
exhibit the lowest coagulation indexes among all the samples because of its coagulation property, but its BCI values at 30 min and 60 min were both above that of 2036 nm, 2738 nm and 3442 nm. This was because the pores sizes of 2036 nm, 2738 nm and 3442 nm were too large, compared with the sizes of the blood cells. In figure 6, besides the red blood cells that had coagulated, it was clear to us that plenty of red blood cells had infiltrated into the pores of these samples, implying that there were less red blood cells released into the supernatants, which would result in lower coagulation indexes. Among the three samples, the BCI values were always close, and there were no significant differences, except for 2036 nm and 3443 nm at 60 min. However, after comprehensive analysis, we could always find that the BCI values tapered with an increase of the fiber diameters, although the difference values between them were small, even with no difference. Therefore, we speculated that the anticoagulation property improved with a decrease of the fiber diameters. Moreover, the fibers of 1456 nm could also induce few red blood cells’ retention within the pores (figure 6), but we believe that its influence on the coagulation indexes was negligible, so it was ignored in the present study.

3.4. HUVEC behaviors on different aligned electrospun fibers

3.4.1. HUVEC adhesion and proliferation

HUVECs were cultured for 6 h to evaluate cell adhesion. As shown in figure 7(A), 73 nm showed the most HUVEC adhesion, up to 20 002 cells cm$^{-2}$. In contrast, the number of HUVECs adhered on 2738 nm was the lowest, only 12 967 cells cm$^{-2}$. For other samples of 582 nm, 1456 nm, 2036 nm and 3442 nm, the adhered cell densities were 17 538 cells cm$^{-2}$, 18 144 cells cm$^{-2}$, 14 835 cells cm$^{-2}$, 14 335 cells cm$^{-2}$, respectively. While comparing all the fibers, there was only a significant difference between 73 nm and 2738 nm, meaning that 73 nm was more suitable for HUVEC adhesion than
2738 nm. Though the difference values between the varying fibers were high, such as 73 nm and 3442 nm, there was no difference between them. Here, we thought that the changes in fiber diameter and roughness had no significant influence on HUVEC adhesion. Our results were in agreement with the conclusions of Dong et al, who found that there were no significant differences among different surfaces (smooth surface, electrospun fibers of 0.61 ± 0.19 μm and 9.90 ± 0.48 μm) on the number of adhered HUVECs per field at 6 h, though their electrospun fibers were random [55]. It is believed that proteins’ adsorption onto biomaterial surfaces is the first event in the biological environment, then cellular interaction [32, 64], meaning that protein adsorption would affect cell adhesion and other behaviors. In the present study, 73 nm attracted the least BSA adsorbed among all the samples, which is significantly lower than 2738 nm and 3442 nm. We believe that more BSA adsorption of 2738 nm and 3442 nm might restrict the cell adhesion-related proteins (fibronectin and hyponectin) adsorbed, leading to fewer HUVECs adhered onto them but more HUVECs onto 73 nm. Furthermore, the hydrophobicity and nano-topography of surfaces could affect the conformation of proteins [54, 60]. Miller et al found that the nano-rough surface could change the conformation of adsorbed fibronectin, resulting in reduced binding sites for cell adhesion [65]. The study of Lord et al showed similar conclusions [61]. HUVEC adhesion is a complex and dynamic process regulated by all the above factors as well as surface area, and these factors are interrelated.

As shown in figure 7(B), the HUVEC on all fiber samples exhibited a similar pattern of time-dependent increase with the progress of the culture period. HUVEC adhesion on 73 nm was significantly better than on 2738 nm at 6 h (figure 7(A)), but there was no difference between the viability values of HUVEC adhered on them at 1 d (figure 7(B)). The absorbance value of 3442 nm was higher than that of all other fiber samples with a significant difference at 1 d. We speculated that the reason might be the large pores of 2036 nm, 2738 nm and 3442 nm, which were large enough for HUVEC infiltration, and the CCK-8 assay would reflect the amount of HUVECs both on the sample surfaces and beneath the surface. For 1456 nm, the HUVEC viability exhibited the second highest absorbance value at 1 d, and we did not find the phenomenon of HUVEC infiltration into the fibers in the present study, so thought that the topography of 1456 nm might benefit for HUVEC adhesion and proliferation during the 1 d culture period. However, the previous study reported that HUVECs could infiltrate into the electrospun scaffold (fibers diameter about 1200 nm) [30]. In our opinion, the fiber density might be responsible for the two opposite results. The fiber density of 1456 nm we fabricated was too small to allow HUVEC infiltration.

At 3 d, there were no differences in the absorbance values among all the fiber samples, meaning that the amounts of HUVEC on varying fibers were approximate. With the extension of the incubation period, the proliferation of HUVEC on different fibers appeared in several trends. At 5 d, the absorbance value of 1456 nm was the highest up to 1.3864 ± 0.1035, then 73 nm of 1.3460 ± 0.0961 and 3442 nm of 1.3406 ± 0.0935. Except for 2738 nm (1.2114 ± 0.0742), the value of 582 nm (1.0633 ± 0.1455) was lower than all the other fiber samples with a significant difference. According to our previous study [32], the finer fiber of 54.77 nm, which mimicked the ultrastructure of the vascular basement membranes, could facilitate ECs’ proliferation significantly better than the thicker fiber of 544.64 nm after culturing for 5 d. Here, we obtained a similar conclusion that HUVEC proliferation on 73 nm was better than on 582 nm, though the fibers were aligned. In the present study, HUVEC would not infiltrate into 582 nm, so we hypothesized that the topography of it could not facilitate HUVEC proliferation like other fibers after a prolonged culture period. We found that 2738 nm also showed a reduced HUVEC proliferation at 5 d. In consideration of cell infiltration, fewer HUVECs adhered on the 2738 nm than on 582 nm. For 2036 nm and 3442 nm, HUVEC adhered onto the fiber surfaces and infiltrated into the gaps of fibers, both
According to ward migrating into the pores and gaps of HUVECs could not among all samples, also relatively high on 3442 nm. We of dead HUVECs on 2036 nm appeared to be the largest (number of living HUVECs was found on 582 nm 2738 nm, as well as on 582 nm. However, the smallest differences on the number of living and dead HUVECs located on the same plane because of the in

Most of the HUVECs adhered on 3442 nm were not adhered onto the surfaces of 73 nm and 1456 nm in the present study, which signified a similar capacity to promote endothelialization (the formation of monolayered ECs) between them. Li et al reported that good EC proliferation could promote rapid cell–cell junction and endothelialization [66].

3.4.2. HUVEC viability and morphology
The live/dead viability kit was applied to assess HUVEC viability on different fiber diameters at 5 d. As shown in figure 8, relatively few dead HUVECs were observed on 2738 nm, as well as on 582 nm. However, the smallest number of living HUVECs was found on 582 nm (figure 7(B)), so we could not reach the conclusion that HUVEC showed better viability on 582 nm as on 2738 nm. It can be seen from figure 8 that the number of dead HUVECs on 2036 nm appeared to be the largest among all samples, also relatively high on 3442 nm. We thought the reason might be that HUVECs could not find sufficient adhesion sites while infiltrating or downward migrating into the pores and gaps of fiber samples. According to figures 7 and 8, there were no significant differences on the number of living and dead HUVECs between 73 nm and 1456 nm, meaning that the impacts of these two fibers on HUVEC viability were similar. Most of the HUVECs adhered on 3442 nm were not located on the same plane because of the infiltration of HUVECs, leading to the unclear HUVEC morphology (figures 8 and 9). The same event also occurred to 2036 nm and 2738 nm, but we found that less HUVEC infiltrated into 2026 nm and 2738 nm than 3442, and the reason was their smaller pore sizes restricted the downward migration of HUVECs. Also, the HUVECs cultured on them were scattered. In contrast, the immunofluorescence images of figures 8 and 9 suggested that HUVECs on 73 nm distributed uniformly and showed better HUVEC cell–cell contact and junction formation than other fiber samples. HUVEC cell–cell contact and junction formation could be seen in 1456 nm as well, but HUVECs on it exhibited a more elongated morphology than on 73 nm (figure 9), also a smaller HUVEC spreading area (figure 10(A)). Dejana had confirmed the critical roles of EC junctional structures on the formation of a functional endothelium barrier and the integrity of the EC monolayer [67].

The surface roughness of electrospun membranes that could affect cell coverage on them increased with the growth of fiber diameter [32, 53, 55, 68]. We found that 73 nm with the smallest roughness demonstrated the most effective coverage by HUVECs in the present study. Also, 582 nm should have performed better than 1456 nm; however, it was worse because of poor HUVEC adhesion and proliferation on it.

In figure 9, it can be clearly found that HUVECs were elongated along the direction of the long axis of all the aligned fiber samples, and the changes in morphology of HUVECs affected their spreading areas. As shown in figure 10(A), HUVECs on 3442 nm got the largest spreading area of 1179.54 ± 380.66 μm², then 73 nm of 1075.77 ± 448.95 μm² and 2738 nm of 1041.07 ± 404.38 μm². For other fibers, the values of 1456 nm, 582 nm and 2036 nm were 935.35 ± 293.60 μm², 875.81 ± 291.37 μm² and 803.75 ± 297.83 μm², respectively, arranged in descending order.
adhered on 73 nm spread well and showed the minimum degree of elongation among all the fiber samples (figures 10(B), (C)), corresponding to a relatively high spreading area. In figure 10(B), the length values of 2036 nm and 2738 nm were close with no significant difference, so were 582 nm and 1456 nm. With an increase of fiber diameter, the long axis lengths (nuclei) of HUVECs grew gradually, exhibiting the largest values on 2036 nm and 2738 nm, then decreasing on 3442 nm. For the nucleus aspect ratio, the values rose successively with an increase of fiber diameter from 73 nm to 2738 nm, reaching the peak value on 2738 nm and then decreasing on 3442 nm (figure 10(C)). The fiber diameter of 3442 nm was too thick for HUVECs to perceive its topography, so it had less influence on HUVEC elongation. Taking into consideration the morphology parameters of HUVECs on different fibers, we inferred that the elongation of HUVECs would confine cell spreading, corresponding to the relatively small spreading area, in agreement with the viewpoints of Chen et al [18, 37]. According to our results, the aligned fibers with diameters around 2036 nm and 2738 nm contributed to the elongation of HUVECs more strongly than other fibers. However, compared to 2036 nm, 2738 nm had less influence on HUVEC spreading and presented a higher nucleus aspect ratio, so we speculated that the contact guidance effect of 2738 nm on HUVEC elongation was more active, and more worthwhile for further intensive exploration.

Additionally, the HUVECs on 73 nm were close to the natural state in native tissue, exhibiting a more similar cobblestone-like morphology than other fiber samples (figure 9). However, several reports had confirmed that elongated ECs showed a better capacity for anti-thrombosis [18, 71] and anti-inflammation [24], as well as more excellent resistance to detachment [14, 15, 30, 66, 72] compared to non-elongated ECs. Therefore, we cannot decide which kind of fiber has more potential to promote the formation of a functional endothelium simplistically based on our limited evaluation on HUVEC behaviors.

3.4.3. NO release

For vascular grafts, it should be noted that improving endothelialization is not enough, and competent and healthy function of regenerated endothelium is also crucial. Among a variety of endothelial functions, anti-thrombosis, which is of benefit for diminishing the
incidence of late graft thrombosis, is one of the vital properties for regenerated endothelium, regulated by many functional molecules [18, 49, 51, 73]. Several research studies have confirmed that an oriented EC monolayer shows better anti-thrombotic property while compared with nonaligned endothelium. However, the reasons for that are unclear and are still being explored. In the blood vessels, nitric oxide (NO) secreted by healthy ECs continuously plays a significant role in preventing platelet adhesion and aggregation, as well as vascular smooth muscle cells over-proliferation [74]. Dong et al found that the NO release of ECs on a smooth surface was significantly higher than electrospun substrates with different fiber diameters because of the rapid formation of an intact and stabilized EC confluent monolayer, and there was no difference on the NO release between these two electrospun substrates (fiber diameters of 0.61 ± 0.19 μm and 9.90 ± 0.48 μm) [55]. However, the electrospun fibers in their study were random, and only two kinds of fiber were assessed. Here, we further evaluated the NO production of HUVECs on aligned electrospun substrates with varying fiber diameters.

As shown in figure 11, 73 nm showed the biggest absorbance value among all the samples, then 2036 nm. Their absorbance values were significantly higher than 2738 nm, which exhibited the lowest absorbance, indicating the most impoverished NO release property of it. We found that 582 nm and 3442 nm performed similarly, relatively higher than 1456 nm and 2738 nm. According to the study of Chen et al, compared with HUVECs cultured on flat film, the higher concentration of PGI2 and tPA released by an oriented EC monolayer restrained platelet aggregation more effectively, leading to a better anti-thrombotic property [18]. Thus, we had reason to hypothesize that HUVEC morphology also contributed to the NO production of HUVEC. HUVECs on 2036 nm and 2738 nm showed similar elongated morphology, while significantly different NO production. For 73 nm and 2036 nm, the performance of NO release was close, yet showed different HUVEC morphology. As shown in figures 8–10, HUVEC morphologies on 582 nm, 1456 nm and 3442 nm were varied, but the amounts of NO release exhibited no difference. Importantly, it should be noted that the number of HUVECs and the NO production capacity of single-cell combined to determine the absorbance value that we measured in the present study, the actual amount of NO released per unit volume of culture medium. From our results, we could only confirm that HUVECs cultured on 73 nm and 2036 nm secreted more NO than 2738 nm with a significant difference, showing their superior anti-thrombotic property during the 2 d culture. Nevertheless, the positive or negative impact of HUVEC elongation on its NO release capacity could not be found. Detailed analysis on the relationship between HUVEC morphology and NO production capacity will be performed in future investigations.

3.5. The viability and TNF-α release property of a RAW 264.7 cell on different aligned electrospun fibers

For vascular implant design it is essential to consider an important requirement, i.e. preventing an inflammatory reaction in the early stages of implantation [42, 75]. During inflammation, blood monocytes could migrate into the tissue and transform into macrophages [44]. Reports have confirmed that inflammation induced by implantable devices might cause thrombosis, hyperplasia, or other serious complications [41]. When macrophages adhere onto biomaterials, inflammatory cytokines are quickly secreted within 24 h [76], so in our experiments, the viability of macrophages at 24 h was regarded as an indicator to evaluate the anti-inflammation property of different fiber samples. Meanwhile, it is widely accepted that TNF-α could stimulate an inflammatory reaction [41, 42]. Thus, we measured the amounts of
released THF-α to assess the activation of macrophages at 24 h. In the present study, we aimed to evaluate the anti-inflammation property of different aligned electrospun fibers, and provide the basis for further implant design.

In figure 12(A), we categorize the absorbance values into three levels roughly, representing varied RAW 264.7 cell viability on different fiber samples. We found that 73 nm, as the high-level, obtained the highest absorbance value, then the group of 2036 nm, 2738 nm and 3442 nm as the medium-level, 582 nm and 1456 nm composing the group of the low-level. The differences between the three groups had statistical significance, while no significant differences were found between the fiber samples of every single group. The concentrations of TNF-α produced by RAW 264.7 cells are shown in figure 12(B). Here, 1456 nm exhibited the lowest concentration of 396.32 ± 46.44 pg ml⁻¹, 27.57% lower than that of 2036 nm (547.14 ± 26.94 pg ml⁻¹), while with no significant difference between them. The highest concentration appeared in 3442 nm (658.64 ± 21.79 pg ml⁻¹), then 2738 nm of 631.88 ± 84.86 pg ml⁻¹, 73 nm of 609.17 ± 51.60 pg ml⁻¹ and 582 nm of 585.66 ± 146.78 pg ml⁻¹, respectively. The differences between the amounts of released TNF-α of RAW 264.7 cells on these four fibers had no statistical significances, but their TNF-α productions were all significantly more than that of 1456 nm.

According to our results, we could see that the RAW 264.7 cells adhered onto 73 nm showed significantly higher viability than onto the fibers of the medium-level group (2036 nm, 2738 nm and 3442 nm) and the low-level group (582 nm and 1456 nm). Also, 73 nm presented relatively high TNF-α production, and its concentration was approximately 1456 nm. Both of these two inflammation-related indicators suggested the worst anti-inflammation property of 73 nm. Besides, RAW 264.7 cell viability on 582 nm and 1456 nm was similar, poorer than all the other fibers, indicating their better anti-inflammation capacity. Comparing these two fibers, significantly higher TNF-α production of 582 nm would weaken its anti-inflammation capacity, so 1456 nm exhibited the best performance in the present study. Additionally, for the fibers of the medium-level group (2036 nm, 2738 nm and 3442 nm), they performed similarly on the RAW 264.7 cell adhesion and TNF-α production. We speculated that the cause was their large fiber diameters, which had exceeded the active size range of the contact guidance effect for a RAW 264.7 cell. Here, we evaluated the amount of TNF-α production on different fiber samples, but could not conclude the impacts of topographies of fibers on the TNF-α release capacity of a single RAW 264.7 cell.

In our experiments, the hydrophobicity, topography, and other surface properties of fiber samples affected the proteins’ adsorption and also changed the conformations of the adsorbed proteins. The denatured-proteins could be recognized by the host immune system, provoking inflammation [77]. Meanwhile, the receptor–ligand interactions between diverse adsorbed proteins and adherent macrophages were varied, which could affect the types and levels of cytokines secreted [78] and further macrophage phenotypes transformation [79]. The release of cytokines and macrophage phenotypes transformation are interrelated [42, 78, 79].

4. Conclusions

In this study, varying diameters of aligned electrospun fibers, ranging from dozens of nanometers to several micrometers, were fabricated to elucidate their impacts on hemocompatibility, HUVEC behaviors and RAW 264.7 cell behaviors. The results showed that the topographies of the varying fibers affected PBS contact angles and BSA adsorption differently, which related closely to the hemocompatibility and cell behaviors, and we have revealed (partly) the relationship between them. Collectively, this work provides

Figure 12. (A) The viability of a RAW 264.7 cell, and (B) the expression of TNF-α released by adhered RAW 264.7 cells after 24 h incubation on different aligned electrospun fibers.
fundamental information for surface topography design of vascular implants to realize varied requirements, such as enhancing endothelialization, as well as restricting the incidences of complications.

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Conflicts of interest

The authors declare no conflict of interest.

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