

Full Length Article

Dual surface modification of poly(L-lactide) scaffold achieved by thermal incorporation of aligned nanofiber and click immobilization of VEGF to enhance endothelialization and blood compatibility

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ABSTRACT

Rapid endothelialization is very important in the development of blood-contact biomaterials because of the inhibitory effects of endothelium in preventing thrombosis and restenosis. In this study, topographical and biochemical cues were generated to accelerate endothelialization on the poly(L-lactide) (PLLA) scaffold via a simple, bio-orthogonal, and biocompatible process. Topographical guidance was incorporated into a PLLA scaffold via thermal attachment of aligned PLLA nanofibers using electrospinning. Vascular endothelial growth factor (VEGF) was covalently immobilized onto the PLLA scaffold by aminolysis and copper-catalyzed azide/alkyne cycloaddition-mediated click chemistry. Various surface analyses confirmed the presence of aligned topography and immobilized VEGF without delamination or leaching. *In vitro* experiments with human coronary artery endothelial cells revealed that proliferation, CD31 expression, and migration was significantly enhanced on the nanofiber-attached VEGF-immobilized PLLA scaffold. The aligned nanofiber also accommodated the sustained release of heparin, which suppressed the proliferation of human coronary artery smooth muscle cells and platelet activation without affecting surface endothelialization. The heparin-loaded, aligned, nanofiber-attached VEGF-immobilized PLLA scaffold was evidenced to be biocompatible and a promising candidate for blood-contact materials where fast endothelialization and suppressed platelet activation is required.

1. Introduction

Polymer-based blood-contacting medical devices, such as stents, artificial vascular grafts, catheters, and sutures, have been widely used in medical practice [1]. poly(L-lactide) (PLLA) is a biodegradable polymer that is commonly used for stents and tissue engineering vascular grafts owing to its suitable physical properties and minimal inflammation *in vivo* [2–7]. However, the use of PLLA in blood contact devices has a number of side effects, including thrombosis and intimal hyperplasia [8].

The most promising solution to these side effects is the promotion of endothelium formation on the surface of the PLLA-based medical device. The endothelium formed on the surface secretes anti-thrombotic molecules and plays a critical role in suppressing the proliferation of smooth muscle cells through a selectively permeable barrier [9,10]. The efficacy of such methods relies on the speed and robustness of endothelium formation at the PLLA surface.

Various physical or chemical surface modifying strategies that promote endothelialization on PLLA-based biomaterials have been investigated [11,12]. The most popular physical modification is based on surface topography. Several studies reported that cell adhesion, migration, alignment, and shape can be regulated by topography [13–15]. In particular, an aligned topography promotes fundamental aspects of endothelialization, including endothelial cell migration and elongation of cell morphology to minimize shear stress [16,17]. Aligned topography can be produced in many different ways. A femtosecond laser can be used to generate an aligned topography, with exact precision, that promotes re-endothelialization [18–20]. However, it requires considerable time-investment and complex procedures. Alternatively, photolithography can be used [21–23], which is a highly efficient and cost-effective method, but it can only be applied to flat substrates, making it less suitable for structurally complex blood contact devices. Another method is nano-imprinting [24–26], which has the advantage of high-throughput but the disadvantage of using expensive master molds. A

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non-patterning method that can generate aligned PLLA-based biomaterials is electrospinning, which can produce aligned nano/micro-fibers. Although electrospinning is efficient and relatively inexpensive, aligned fibrous scaffold is mechanically weak or difficult to manipulate and requires perfect sealing techniques to function in blood contact devices. In addition to the disadvantages of the various methods mentioned above, a common limitation is that aligned topography only provides passive topographical cues without any active functions.

Biochemical modification that promotes endothelialization is aimed at covalently attaching various bioactive molecules to the device surface. Vascular endothelial growth factor (VEGF) is commonly used as a biochemical cue due to its upregulation of factors essential to endothelialization [27,28]. VEGF can be attached to biomaterial surfaces via pre-grafted polymer, polydopamine, and other linkers [29–31]. Most frequently, primary amine groups in VEGF are reacted with functional groups on the device surface by EDC/NHS chemistry or glutaraldehyde treatment. These conventional conjugation reactions are generally non-bioorthogonal, which could potentially affect the native tissues or interfere with the biological processes of various microenvironments. Therefore, bioorthogonal surface immobilization techniques that can selectively react with target bioactive molecules are necessary to improve biosafety by preventing unwanted reactions. Although VEGF has been incorporated onto PLLA surfaces, most studies applied physical coating [32] and only a few of them used covalent immobilization [33,34]. This is because reactive functional groups are not inherent to PLLA. There have been no attempts to use bioorthogonal conjugation, like click chemistry, in covalently immobilizing VEGF on PLLA surfaces.

In this study, we proposed novel dual surface modification strategies to promote surface endothelialization on a PLLA-based substrate. Both topographical and biochemical cues were incorporated into PLLA substrates. First, to provide topographical cues to endothelial cells, the horizontally aligned PLLA electrospun nanofiber was thermally attached to the surface of a PLLA substrate. The method is more cost-effective and less time-consuming than conventional printing techniques and does not require expensive equipment, complicated procedures, or chemical treatment. The biochemical cue was bioorthogonally conjugated to the PLLA substrate by covalent immobilization of VEGF using click chemistry called as copper-catalyzed azide/alkyne cycloaddition (CuAAC). The click reaction is biocompatible and highly selective, so unreacted functional groups are less likely to cause complications *in vivo*. After preparation of PLLA substrates by incorporating both topographical and biochemical cues, *in vitro* studies were performed to demonstrate the effects of both cues on the proliferation, CD31 expression, and migration of human coronary artery endothelial cells (HCAECs). Furthermore, the aligned fibers were loaded with heparin and attached to PLLA substrates to evaluate their drug delivery functions, specifically about anti-proliferative efficacy on the human coronary artery smooth muscle cells (HCASMC) and improving blood compatibility.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and solvents were of analytical grade and used as provided by the manufacturers. PLLA (Mw = 200,000) was purchased from BMG, Inc. (Kyoto, Japan). Azido-poly(ethylene glycol)5 -N-hydroxysuccinimidyl (Azido-PEG5-NHS) ester was purchased from BroadPharm (San Diego, CA, USA). 1,6-Hexamethylenediamine (HDA), propargyl-N-hydroxysuccinimidyl ester, copper (II) sulfate, tris(3-hydroxypropyl-triazolylmethyl)amine, (+)-sodium L-ascorbate, bovine serum albumin (BSA), and heparin sodium salt from porcine intestinal mucosa were purchased from Sigma Aldrich (Milwaukee, WI, USA). Human VEGF-A165 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Endothelial basal medium-2 (EBM-2) and Smooth Muscle Cell Growth Medium-2 (SMGM-2) were purchased from Lonza Group Ltd. (Basel, Switzerland). 3-(4,5-

Dimethylthiazol-2-yl)diphenyltetrazolium bromide (MTT) was purchased from Invitrogen Corp. (Carlsbad, CA, USA).

2.2. Fabrication of fiber-attached PLLA scaffold

Aligned PLLA nanofibers were prepared using an electrospinning technique. First, the polymer solution was prepared by dissolving PLLA in chloroform/DMF (8:2, v/v) at a concentration of 8 wt%, according to a previous study [35]. The solution was loaded into a 10 mL syringe with a 25 G (0.26 mm inner diameter) conducting needle connected to the positive terminal of an electrospinning instrument. An emitter needle applied 14 kV to the solution, which was supplied at a constant feed rate of 0.4 mL/h. An aluminum foil was attached to a drum spinning at 960 rpm for collecting the fibers. The distance between the needle and the drum was 10–15 cm. After 40 min of electrospinning, the foil was detached from the drum and cut into 1 × 1 cm pieces.

PLLA film was obtained using a drop-casting method, where 100 μ L of 5 wt% PLLA in chloroform solution was drop-casted on a 1 × 1 cm silicon wafer. The PLLA film was then dried at room temperature for 24 h.

Fiber-attached PLLA scaffold was obtained by thermally attaching PLLA nanofiber to the film. The PLLA film on the silicon wafer was heated at 135 °C for 3.5–4.5 min and attached to PLLA fibers by applying a pressure of 9.8 kPa. The fiber-attached scaffold was submerged in water and peeled from the silicon wafer using tweezers.

2.3. Immobilization of VEGF on the scaffold

Coupling of an azide group to VEGF was performed by sulfo-NHS-ester reaction [36,37]. After dissolving VEGF in phosphate buffered saline (PBS) (1.0 mg/mL, 0.025 mM) and Azido-PEG5-NHS ester in PBS (13.5 mg/mL, 31.27 mM), 100 μ L of VEGF solution was mixed with 4.1 μ L of Azido-PEG5-NHS ester solution (50-fold molar excess of VEGF). The reaction occurred at 4 °C for 2 h. To remove unreacted Azido-PEG5-NHS ester, the mixed solution was then concentrated by ultrafiltration in a Pierce protein concentrators PES 10 kDa MWCO (Thermo Fisher Scientific, Waltham, MA, USA). The azide-conjugated VEGF solution was diluted in PBS to make 64 μ g/mL (0.0016 mM).

To attach functional groups to PLLA fiber scaffolds, aminolysis was performed first as previously described [38]. In brief, PLLA fiber scaffolds were immersed in 1 mg/mL (0.516 M) HDA/isopropanol solution at 50 °C for different reaction times (0, 10, and 40 min). The PLLA fiber scaffolds were washed with isopropanol followed by deionized water and dried. Through various analyses, we qualitatively confirmed the formation of amino groups on scaffolds after aminolysis at the set times. Scaffolds were treated with 200 μ L of a 10 mg/mL fluorescein isothiocyanate (FITC)-labeled-NHS ester (Thermo Fisher Scientific) in DMSO solution for 1 h at room temperature. After the scaffolds were washed with deionized water and dried, fluorescence images and intensities were obtained using an inverted fluorescence microscope (IX53, Olympus Corp., Tokyo, Japan).

Aminolyzed PLLA fiber scaffolds were then coupled with a propargyl group. The scaffolds were immersed in 0.043 mg/mL (0.19 mM) propargyl-N-hydroxysuccinimidyl ester and dimethyl sulfoxide (DMSO) solution at room temperature for 30 min with gentle shaking. The scaffolds were then washed twice with deionized water.

After conjugation of the azido group to VEGF and alkyne group to the PLLA scaffold, a CuAAC reaction was carried out to covalently immobilize VEGF onto the scaffold surface. Each sample was treated with 49 μ L of 64 μ g/mL azide-coupled VEGF solution and 1 μ L of catalyst solution and incubated for 1 h at room temperature. The surfaces were then washed twice with PBS and dried. The catalyst solution contained 0.8 mg/mL (0.005 M) of CuSO₄, 10.85 mg/mL (0.025 M) of tris(3-hydroxypropyl-triazolylmethyl) amine, and 39.6 mg/mL (0.2 M) of (+)-sodium L-ascorbate. The experimental procedure is described in Fig. 1, and sample abbreviations are summarized in Table 1. Unless

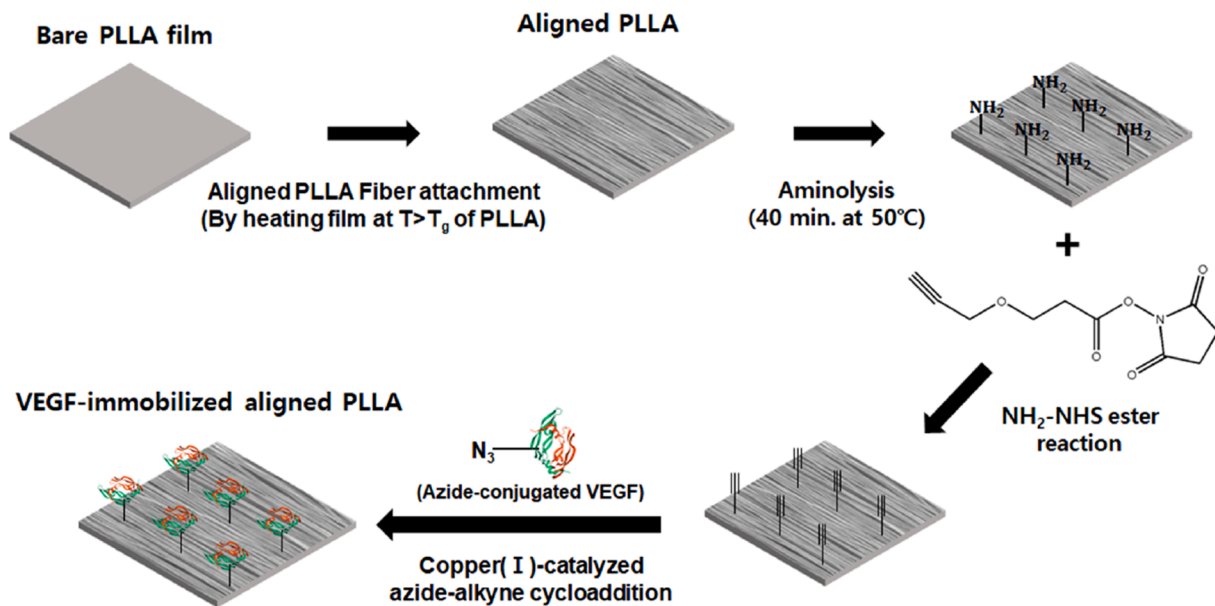


Fig. 1. Schematic illustration of dual modification of PLLA scaffold by incorporating aligned nanofiber and VEGF.

Table 1
Sample abbreviations and their characteristics

Sample name	Morphology	VEGF immobilization	Heparin loading (52.5 mg heparin/g PLLA fiber)
Bare	Flat	–	–
Aligned	Aligned	–	–
V-Bare	Flat	Covalently bonded	–
V-Aligned	Aligned	Covalently bonded	–
V(P)-Bare	Flat	Physical attachment	–
V(P)-Aligned	Aligned	Physical attachment	–
V(S)-Aligned	Aligned	Dissolved in media	–
H-Aligned	Aligned	–	Loaded
VH-Aligned	Aligned	Covalently bonded	Loaded

otherwise stated, PLLA scaffold represents nanofiber-attached PLLA films, while PLLA nanofibers and bare PLLA scaffolds without fibers were expressed as PLLA nanofibers and PLLA films, respectively.

2.4. Characterization of surface properties

Scaffold morphology and surface roughness were investigated using field-emission scanning electron microscopy (FE-SEM) and atomic force microscopy (AFM). SEM images were obtained using a JEOL-7610F-Plus at 10 kV (JEOL Ltd., Tokyo, Japan) and AFM images were obtained using an NX20 (Park Systems, Suwon, Korea) on a surface area of 200×200 nm. X-ray photoelectron spectroscopy (XPS) spectra were recorded on a K-alpha (Thermo Fisher Scientific) using Al K α excitation radiation (1486.6 eV, sampling area; 60×60 mm). Survey spectra were collected using a pass energy of 200 eV with a step size of 1.0 eV. The charging shift was interpreted using the N1s spectrum.

2.5. Quantification of immobilized VEGF

Several methods have been used to verify the immobilization of VEGF. First, the VEGF-immobilized samples were incubated with Alexa flour 488-labeled anti-VEGF (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions. The samples were subsequently washed five times with 0.05 wt% tween-20/PBS solution to remove

unreactive anti-VEGF and observed with fluorescence microscopy. Next, a direct enzyme-linked immunosorbent assay (ELISA) technique was used to quantify immobilized VEGF according to a human VEGF ELISA kit (ab100662, Abcam, Cambridge, UK) protocol with minor modification. Prior to the ELISA reaction, the VEGF-immobilized scaffolds were incubated in 1.0 wt% BSA solution (in PBS, pH 7.4) for 1 h at room temperature to prevent non-specific binding; the scaffolds were then placed in 12-well plates and incubated with a biotinylated detection antibody for 1 h at room temperature. After the surfaces were incubated with streptavidin–HRP for 45 min at room temperature and washed with deionized water, they were reacted with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 30 min at room temperature. The absorbance was measured at 450 nm using a Versamax plate reader (Innotech, Daegu, Korea).

The leaching rate of immobilized VEGF from the scaffold was also investigated. The scaffolds were soaked in 3 mL PBS solution using centrifuge tubes and incubated for 30 d at 37 °C. The solution was replaced by the same volume of fresh PBS every 3 d. The amount of leached VEGF was measured using the VEGF ELISA kit (Abcam).

2.6. Cell culture

Human coronary artery endothelial cells (HCAEC, CC-2585, Lonza Group) were used between passages 6 and 8. Cells were subcultured in EBM-2 under standard culture conditions (37 °C with 5% CO₂, 95% humidity). EBM-2 basal medium (excluding VEGF) was used in all experiments for evaluating the behavior of HCAECs and changed every 3 d.

HCASMC (CC-2583, Lonza Group) were used between passage 6 and 8. Cells were subcultured in SMGM-2 under standard culture conditions (37 °C with 5% CO₂, 95% humidity). SMGM-2 was used in all experiments for evaluating the behavior of HCASMCs and was changed every 3 d.

The scaffolds were placed in 12-well plates and sterilized by UV irradiation for 30 min. The desired cell number was centrifuged into a pellet and resuspended in the medium, corresponding to 50 μ L per PLLA fiber scaffold. The cells were seeded on the scaffold and incubated for 3 h at 37 °C, followed by an addition of 1 mL of fresh culture medium.

2.7. Cytotoxicity evaluation

The cytotoxicity of the PLLA-based substrate was evaluated by MTT

assay and fluorescence live/dead assay. After 1.0×10^4 cells were seeded on each scaffold, the assays were performed according to the manufacturer's protocol. To perform the MTT assay, medium was replaced with MTT/medium solution (0.5 mg/mL), incubated for 1 h at 37 °C, and dissolved in DMSO to obtain purple formazan. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). The fluorescence live/dead assay was conducted using live/dead assay kit (L-7013, Invitrogen). Briefly, the substrates were washed with PBS and placed in the 2%(v/v) live/dead assay solution for 1 h. The fluorescence images were acquired with a fluorescence microscope IX53 (Olympus Corp.).

The activity of azide-functionalized VEGF was also evaluated by MTT assay. After 1.0×10^4 cells were seeded on each well of the 24-well plate, the MTT assays were performed according to the manufacturer's protocol in the same manner as mentioned above. Two mL of EBM-2 basal medium with azide-functionalized VEGF or free VEGF (1000 µg/mL) was changed every 3 d.

2.8. Immunofluorescence staining

For the investigation of CD31 expression, 1.0×10^4 HCAECs were seeded onto 1×1 cm scaffold and cultured for 3 d with EBM-2 (excluding VEGF). Prior to analysis, cells were washed with PBS and fixed with 4% v/v paraformaldehyde for 20 min at room temperature. Cells were then permeabilized with 0.1 wt% triton X-100 (in PBS) for 3 min at room temperature and blocked with 1 wt% BSA (in PBS) for 40 min in an incubator. For immunostaining, the cells were incubated with Alexa Fluor 488-labeled CD31 antibody (Santa Cruz Biotechnology) according to the manufacturer's instructions. After three PBS washes, the cells were incubated with rhodamine-phalloidin (Invitrogen Corp.) for 1 h at room temperature, and then with DAPI staining solution (Invitrogen Corp.) for 5 min at room temperature. The surfaces were washed before fluorescence images were recorded with an LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany) and cell count and fluorescence intensity determined using ImageJ software.

For the measurement of cell orientation, the experiment was conducted in the same manner as above without CD31 immunostaining. The fluorescence images were recorded with an LSM 980 confocal microscope (Carl Zeiss, Oberkochen, Germany). Cell orientation was determined using ImageJ software.

2.9. Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA was isolated from the HCAECs on culture day 3 using an RNA extraction kit (Takara, Japan) according to the manufacturer's instructions. The RNA samples were quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). After synthesizing the complementary DNA using a ReverTra Ace qPCR RT kit (Toyobo, Japan), RT-PCR was performed using a Bio-Rad CFX 96 system (Hercules, CA, USA) with SYBR green mix (Takara, Shiga, Japan). The mRNA expression for the endothelial marker CD31 was normalized by a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase). The primer sequences used for the RT-PCR are presented in (Table 2).

Table 2
The primer sequences used for the RT-PCR

Gene		Primer sequences
CD31	Sense	5'-GGT GGA TGA GGT CCA GAT TTC-3'
	Antisense	5'-GAT GGG ACC AGA TCC TTC ATTC-3'
GAPDH	Sense	5'-CTC CTC ACA GTT GCC ATG TA-3'
	Antisense	5'-GTT GAG CAC AGG GTA CTT TAT TG-3'

2.10. HCAEC migration assay

A rectangular cell-free area was created at the center of the PLLA scaffold by attaching polydimethylsiloxane (PDMS) film (1×10 mm) on the sample and subsequently seeding 1.0×10^4 HCAECs on each separated region of the sample. The PDMS film was removed from the sample after 1 d of incubation and cell migration was monitored for 24 h after PDMS removal. For visualization of cell migration, the cells were stained with calcein-acetoxymethyl ester (Thermo Fisher Scientific). Optical and fluorescence images were obtained using an IX53 inverted fluorescence microscope (Olympus Corp.). Cell coverage by migration was measured using Image J software.

2.11. Heparin loading in PLLA fiber scaffold

A heparin-loaded PLLA scaffold was prepared by electrospinning and thermal attachment using the same procedure described in the previous section. Briefly, 100 µL of 525 mg/mL unfractionated heparin in DMSO solution was mixed with 12.5 g of 8 wt% PLLA solution in chloroform/DMF (8:2), and the resultant solution containing PLLA and heparin was electrospun to obtain heparin-loaded PLLA fiber. These concentrations were chosen based on literature and clinical findings [39,40]. The fibers were attached to PLLA film to obtain heparin-loaded aligned PLLA scaffold, which was then underwent VEGF immobilization process. For the investigation of controlled release of heparin, the scaffolds were soaked in 3 mL PBS solution using centrifuge tubes and incubated for 30 days at 37 °C. The PBS was replaced by an equal volume of fresh PBS every 3 d. The amount of heparin was detected using a heparin ELISA kit (MBS752573, MyBioSource, Inc., San Diego, CA, USA), following the kit protocol.

2.12. Evaluation of platelet adhesion

Platelet adhesion was evaluated to confirm the activity of heparin loaded to the scaffold. Human platelet (Astarte Biologics, Redmond, WA, USA), with a concentration of 2×10^6 U/mL in PBS, were seeded on the scaffold at room temperature for 60 min. After being fixed by 2.5 wt % glutaraldehyde and dehydrated in ascending ethanol/water mixtures, the morphology and number of platelets were visualized by FE-SEM.

2.13. Evaluation of in vivo biocompatibility

All animal experiments were approved by the University Animal Care and Use Committee of the Yonsei University Hospital (approval number: 2020-0236). C57BL/6 seven-week-old mice were purchased from Orient-bio (Seongnam, Korea) and housed in a pathogen-free environment, under a standard 12 h light/dark cycle with food and water provided ad libitum. A total of eight animals were used in the experiment. Four replicates of VH-Aligned scaffolds were implanted into four different mice for histological analyses, and the other four mice were used as a control group. To implant the scaffolds and sham operation, the mice were anesthetized with Alfaxan (76 mg/kg) and xylazine (10 mg/kg) and shaved. After sterilizing the skin with povidone-iodine, a longitudinal incision (2 cm) was made in the central dorsal region to allow access to the subcutaneous space. The UV sterilized scaffold was carefully inserted between the fascia and dermis. For this experiment we immobilized mouse VEGF-A165 (Santa Cruz Biotechnology) to the scaffold. After implantation, the incision was closed with nylon suture. The mice were monitored and weighed for 4 weeks after the operation. During the post-operation period, mice behaved normally with no signs of discomfort.

After four weeks, the scaffold was separated from the surgical site together with the surrounding tissue. The scaffold and tissue were fixed with formalin and placed in paraffin wax. Tissues were cut into 4 µm sections and mounted onto slides that were then deparaffinized with xylene and ethanol. Collagen capsule formations and inflammation

scores were examined by staining with hematoxylin and eosin (H&E). The inflammation score (proportion of cells showing inflammatory signs) was measured using an optical microscope and the scores were defined as: 0 for no inflammatory cells, 1 for mild (0–25% of cells), 2 for moderate (25–75%), and 3 for severe (75–100%).

2.14. Statistical analysis

All measurements presented in graphical form include error bars that reported as mean $\pm$ standard deviation. Each experiment was repeated at least three times. One-way analysis of variance (ANOVA) was used to analyze the differences within each dataset. Tukey's multiple comparison test was used to investigate the variation between the groups. Significance is denoted by $p < 0.05$; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

3. Results

3.1. Surface morphology of nanofiber-attached scaffolds

Fig. 2a and b shows the images of bare PLLA film and aligned PLLA scaffold that was obtained by drop casting and thermal heat treatment, respectively. Bare scaffold was used to imitate the surface of blood contact devices, whereas the aligned fiber was used to provide topographical cues to cells. The fibers were firmly attached to the PLLA film without delamination and formed an effectively integrated scaffold. The scaffold is flexible and can be rolled into a tubular shape, as shown in Fig. 2c.

The aligned PLLA nanofibers were obtained using conventional electrospinning. SEM images confirmed that the fibers, with an average diameter of 776 ± 144 nm, were successfully aligned in a parallel configuration (Fig. 2d). Although fibers were slightly flattened after attachment to the PLLA substrate and subsequent aminolysis, the aligned architecture was well maintained (Fig. 2e and f). Immobilization of VEGF did not cause a significant change of scaffold morphology

(Fig. 2g).

The bonding strength between PLLA nanofibers and PLLA films was also tested by recording the tensile strength using Mecmesin material testers (Mecmesin, Ltd., United Kingdom) (Fig. S1 in Supporting Information). For this, samples were prepared by attaching PLLA nanofibers to PLLA film so that only a half region of the nanofiber overlaps the film as shown in Fig. S1a. Since the bonding strength between the nanofiber and the film was so strong that they did not separate from each other, instead only the nanofibers were broken as shown in Fig. S1b. Regardless of the attached nanofibers' orientation, the fibers did not detach from the PLLA film even when a 100 MPa or more tensile strength was applied. In the case of scaffolds with horizontal and vertical overlapping parts, the maximum tensile strength where the nanofiber part was broken was 156.9 and 196.1 MPa, respectively. These results also confirmed that PLLA nanofibers were firmly attached to the PLLA film via thermal treatment.

3.2. Characterization of scaffold after aminolysis and VEGF immobilization

The aminolysis reaction between HDA and PLLA successfully produced amine groups on the PLLA film surface, confirmed through XPS analysis. Following the completed aminolysis reaction, C—N, N—C=O, and C—NH₃⁺ appeared as a merged peak in the N1s spectra (Fig. 3a). Fig. 3a also shows that the degree of aminolysis was proportional to reaction time, where stronger peak intensities were observed as reaction time increased.

The presence of amine groups on the scaffold surface was additionally confirmed using FITC-labeled NHS, which bonds covalently with amine groups and fluoresces. Fluorescence intensity increased along with aminolysis reaction time, corresponding to an increase of amine groups on the scaffold (Fig. 3b). The XPS and fluorescence analyses confirmed that functional amine groups were formed on the PLLA surface via aminolysis. The amine groups were then reacted with propargyl-N-hydroxysuccinimidyl ester by NHS ester reaction to conjugate alkyne

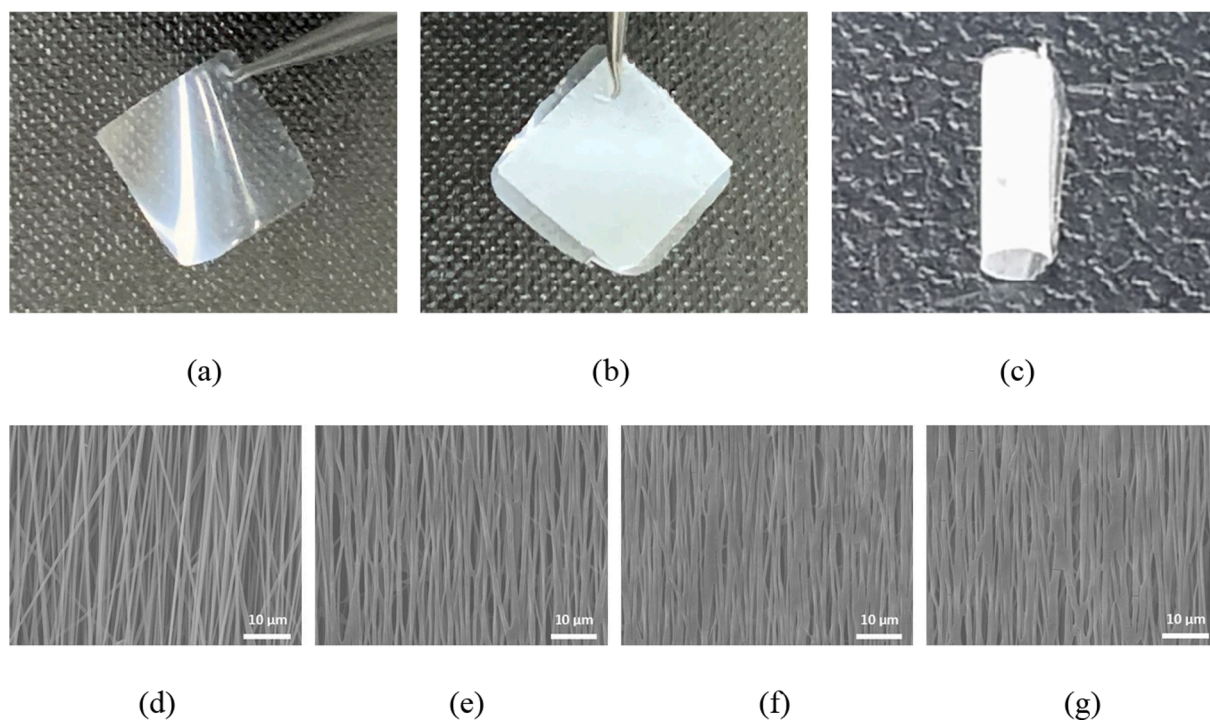


Fig. 2. Photos and SEM images of the different scaffold. Photo of (a) bare PLLA film, (b) PLLA scaffold incorporating aligned PLLA nanofibers, and (c) rolled nanofiber-attached PLLA scaffold. SEM images of (d) aligned PLLA nanofiber obtained after electrospinning and (e) aligned nanofiber incorporated into the PLLA scaffold. SEM images of nanofiber-attached PLLA scaffold underwent aminolysis (f) before and (g) after VEGF immobilization.

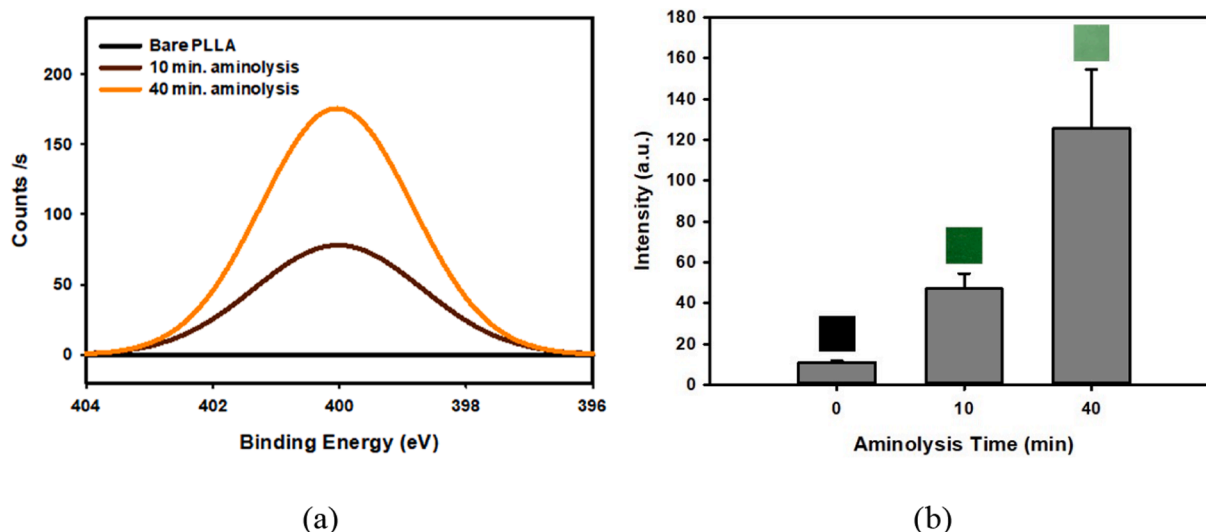


Fig. 3. Characterization of PLLA film after aminolysis reaction. (a) XPS N1s spectra of PLLA film with different aminolysis reaction times. (b) Fluorescence intensity and images obtained after reacting FITC-labeled NHS with PLLA underwent aminolysis for a different time.

groups to the surface. According to a previous study, it was known that the longer the aminolysis time, the greater the fiber damages [38]. In this study, we found that aminolysis longer than 40 min caused significant changes in fiber morphology. For example, fiber diameter was not changed up to 40 min, but it decreased to 49.3% of its original diameter after 60 min aminolysis. Therefore, we fixed the aminolysis time to 40 min in the rest of the experiments.

VEGF was covalently immobilized onto the scaffold via CuAAC reaction between azide groups on VEGF and alkyne groups on PLLA-based scaffold surfaces. To examine the activity of azide-functionalized VEGF, we compared the activity of azide-conjugated VEGF and free VEGF by monitoring their effects on HCAEC proliferation. As shown in Fig. S2 in Supporting Information, there was no significant difference in HCAEC proliferation between the two groups, confirming that chemical modification of VEGF did not significantly affect their function. XPS analysis was conducted to verify the immobilization of VEGFs to PLLA substrate (Fig. 4a). The intensities of N1s peaks significantly increased after immobilization due to the high amount of amide bonds in VEGF. Covalent bonding was confirmed by comparison with samples in which VEGFs were physically absorbed. V(P)-Bare and V(P)-Aligned scaffolds were prepared by incubating unmodified PLLA film and aligned PLLA scaffold in VEGF solution (64 $\mu\text{g/mL}$) for 1 h at room temperature, respectively. The comparison between physical and chemical immobilization confirmed that a larger proportion of VEGF was stably immobilized onto the PLLA scaffold by covalent bonding through CuAAC reaction. The immobilization of VEGF onto aligned PLLA scaffold was further investigated by treatment with Alexa fluor 488-labeled anti-VEGF. Fig. 4b shows the fluorescence intensities of bound anti-VEGF emitted from different samples. Similar to the XPS results, higher fluorescence intensities were observed from the CuAAC reaction samples. Owing to its larger surface area, immobilized VEGF can attach in greater amounts to aligned PLLA scaffold than to bare PLLA film. A direct ELISA assay using VEGF antibody was conducted for precise quantification of immobilized VEGF (Fig. 4c). This analysis confirmed our earlier results, and the amount of VEGF immobilized to the V-Aligned PLLA with 40 min aminolysis was $935 \pm 196 \text{ pg/cm}^2$.

The durability of fixed VEGF was also monitored by recording VEGF leaching from the scaffold over time. There was negligible release of VEGF over 30 d, indicating that VEGF was stably immobilized onto the scaffold surface.

The change of surface morphology by VEGF immobilization was further analyzed using AFM. According to the AFM images (Fig. 5a) and quantitative roughness data (Fig. 5b), the bare scaffold initially had a

smooth surface that became increasingly rough with aminolysis and VEGF immobilization.

The wettability was confirmed by contact angle measurement (Phoenix-MT, Surface Electro Optics, Suwon, Korea). Water contact angle of nanofiber-attached PLLA scaffold decreased from $75.2 \pm 3.5^\circ$ to $29.3 \pm 4.0^\circ$ after VEGF immobilization.

3.3. Proliferation and viability of HCAECs on the scaffold

The effects of immobilized VEGF and aligned architecture on the proliferation of HCAECs were evaluated using the MTT assay.

Significantly higher cell proliferation was observed from VEGF-immobilized sample (Fig. 6a). Regardless of the presence or absence of aligned architecture, very little cell proliferation was observed on scaffolds without immobilized VEGF. To examine the stability of immobilized VEGF, we compared aligned scaffold produced in similar conditions with equal amounts of soluble VEGF (V(S)-Aligned) (Fig. S3 in Supporting Information). HCAEC proliferation on V(S)-Aligned scaffold was much lower than that of V-Aligned scaffold, implying much greater efficiency of surface-bound VEGF over soluble VEGF. No dead cell (red) was found, and only green fluorescence was observed in the live/dead assay, confirming that there was no cytotoxicity of proposed PLLA scaffolds on the HCAECs (Fig. 6b).

The morphology of HCAECs grown on V-Aligned scaffold indicates that the cells were elongated along the aligned nanofibers, confirming the contribution of thermally-attached aligned nanofiber to cellular alignment and elongation via better contact guidance (Fig. S4a in Supporting Information). Additional image analysis reveals that most of the HCAECs were distributed in one direction, within $\pm 30^\circ$ of pattern direction (Fig. S4b and c in Supporting Information).

3.4. CD31 expression of HCAECs on the scaffold

Biological activity of VEGF was investigated by immunofluorescence staining (Fig. 7a and b) and PCR analysis (Fig. 7c) of CD31 expression. CD31 is particularly expressed at the intercellular junction of an endothelial cell and is involved in the maintenance of junctional integrity [30]. Higher expression of CD31 was mainly associated with surface-immobilized VEGF, whereas aligned architecture alone did not induce higher expression of CD31 without immobilized VEGF. Similar to the cell proliferation results, soluble VEGF had little effect on the CD31 expression (data not shown). Equivalent results were obtained from the RT-PCR analysis, where expression of CD31 was maximized on V-

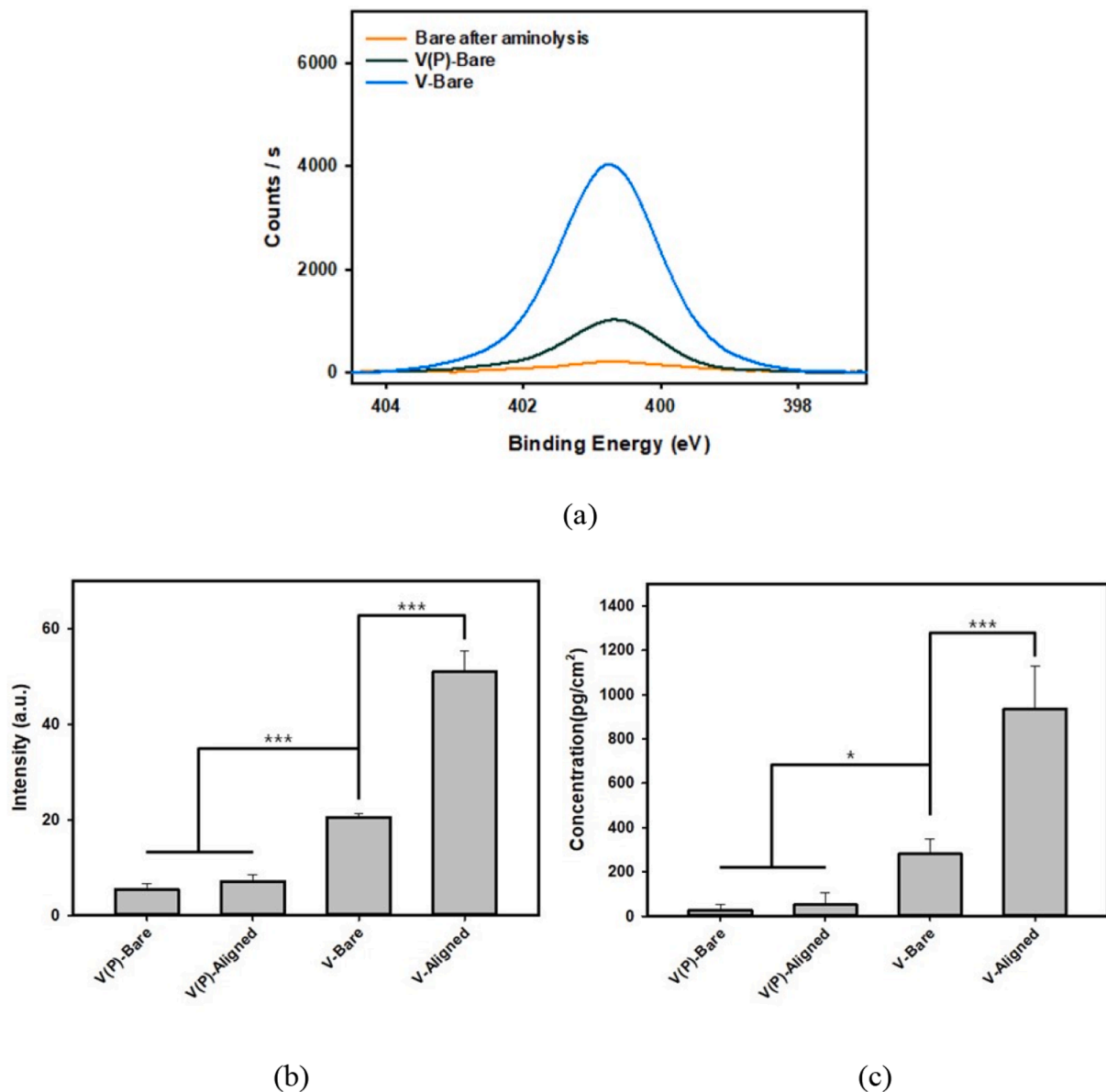


Fig. 4. Analysis of VEGF immobilization. (a) XPS N1s spectra of PLLA film before and after immobilization of VEGF. (b) Fluorescence intensity of Alexa fluor 488-labeled anti-VEGF bound to VEGF immobilized onto different scaffolds. (c) Quantification of VEGF immobilized onto different scaffolds via direct ELISA assay. The symbol *, **, and *** indicate statistical significance to a level of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, using one-way ANOVA with a Tukey post-hoc test, which compares between V(P)-Bare/Aligned and V-Bare groups and between V-Bare and V-Aligned groups.

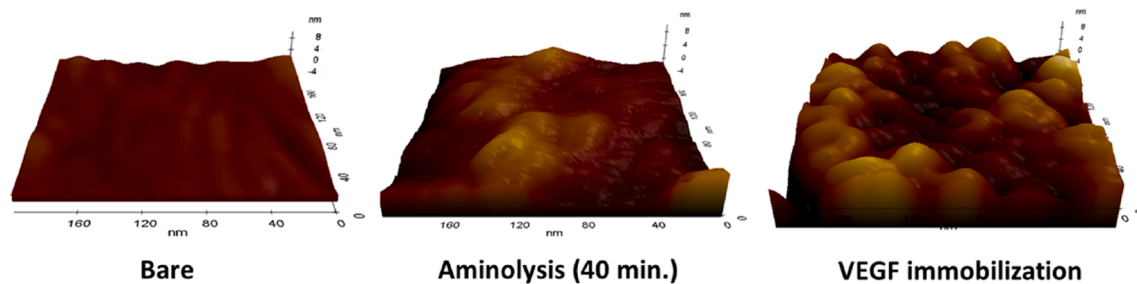
Aligned scaffold (Fig. 7c).

3.5. HCAECs migration assay

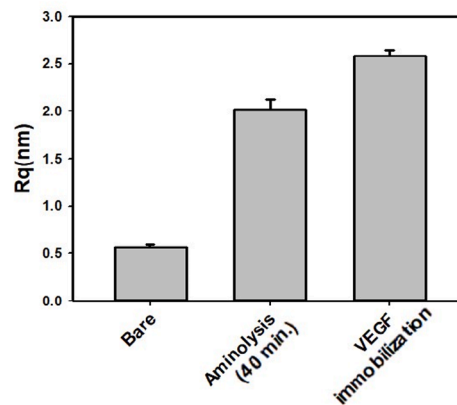
Cell migration was evaluated through coverage ratio, which was defined as the percentage of the area covered by migration of HCAECs after 24 h (Fig. 8a). On the bare scaffold, recovery ratio was only $2 \pm 6\%$, implying limited cell migration. Aligned, V(S)-Aligned, V-Bare, and combined V-Aligned scaffolds induced recovery ratios of $40 \pm 12\%$, $39 \pm 10\%$, $50 \pm 13\%$ and $97 \pm 2\%$, respectively. Soluble VEGF did not affect HCAEC migration (data not shown). These fluorescence results were further confirmed by monitoring migration velocity of HCAECs on different PLLA scaffold, where fastest migration was observed on V-Aligned scaffold (Fig. 8b).

3.6. Incorporation of heparin into aligned fiber

To incorporate additional functions with aligned architecture, heparin was loaded to the fibers during the electrospinning process. The release profile of heparin was divided into two stages (Fig. S5 in Supporting Information). A relatively fast release of heparin took place until day 6, followed by a slow release until day 30. The total amount of heparin release over 30 d was 11.7 wt% of the total loaded heparin ($0.13 \mu\text{g}$ in one scaffold, 0.5 wt% of attached PLLA fiber). Before evaluating the activity of heparin, we confirmed that the release of heparin had a negligible effect on the proliferation of HCAECs (Fig. S6a in Supporting Information). We evaluated the capability of released heparin to inhibit the proliferation of HCASMCs. As shown in Fig. S6b in Supporting Information, proliferation of HCASMCs was suppressed only on the heparin-loaded scaffold, among which VEGF-immobilized scaffold showed greater suppression. The heparin activity was further investigated by evaluating the number and morphology of platelets

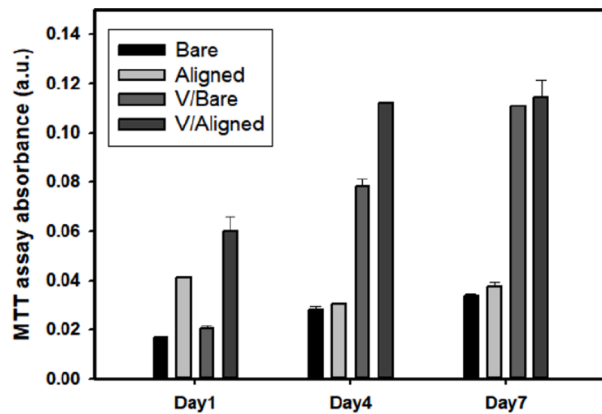


(a)

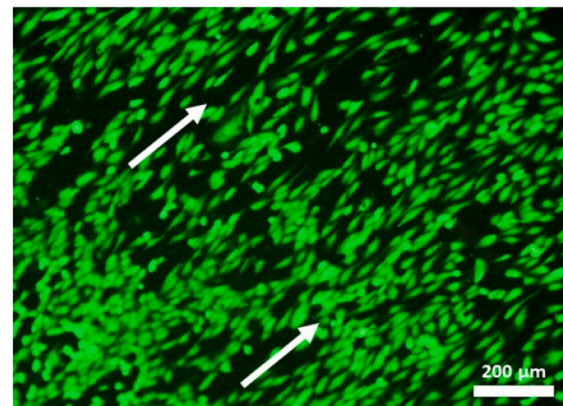


(b)

Fig. 5. Analysis of surface morphology using AFM. (a) AFM height images. (b) Values of root mean square average of height deviation (R_q) obtained from different bare scaffold.



(a)



(b)

Fig. 6. Cytocompatibility of different scaffolds on HCAECs. (a) MTT assay on the proliferation of HCAEC cultured on different scaffolds. (b) Fluorescence live/dead assay on HCAECs cultured on VEGF-immobilized and aligned nanofiber-attached PLLA (V-Aligned) scaffold (arrows indicate the direction of alignment).

applied to the different scaffolds (Fig. S7 in Supporting Information). Similar to the HCASMC proliferation results, the number of adhered platelets significantly decreased by heparin loading (Fig. S7a in Supporting Information). The number of adhered platelets on aligned scaffold ($11,259 \pm 1,208$ platelets/cm²) decreased after either VEGF immobilization ($9,111 \pm 1,114$ platelets/cm²) or heparin loading ($8,025 \pm 873$ platelets/cm²), due to increased hydrophilicity of the

scaffold by VEGF immobilization [41] and release of heparin. Combination of VEGF immobilization and heparin loading further decreased the number of adhered platelet ($4,198 \pm 349$ platelets/cm²).

This indicates that heparin released from the scaffold effectively suppresses platelet adhesion. Platelet morphology is a commonly used criterion for qualitative assessment of platelet activation [42]. As shown in Fig. S7b in Supporting Information, platelets appeared highly

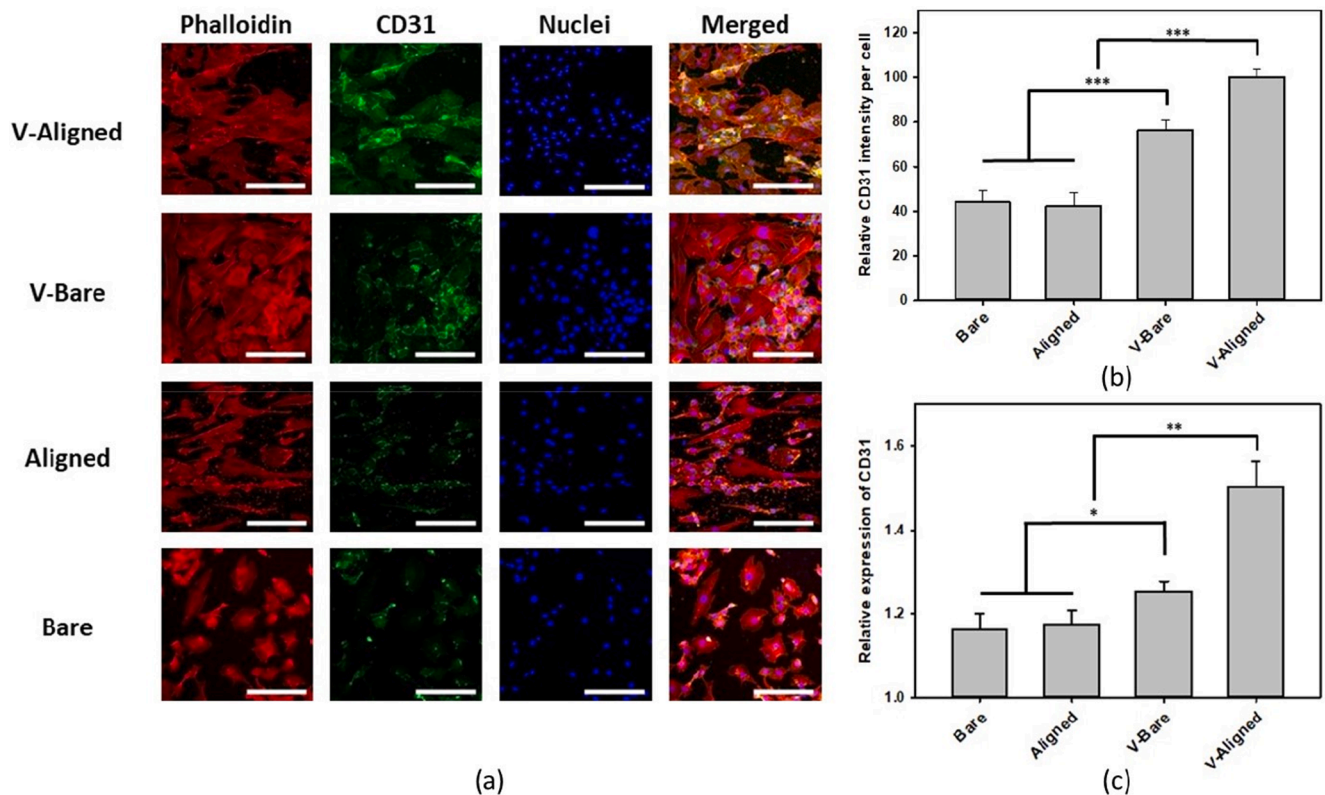


Fig. 7. Study of CD31 expression of HCAECs on the various scaffold. (a) Confocal images (scale bar = 100 μm) and (b) fluorescence intensity of immunofluorescence staining of HCAECs after 3 days of culture. (c) PCR analysis of CD31 expression after 3 days of culture. The symbol *, **, and *** indicate statistical significance to a level of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, using one-way ANOVA with a Tukey post-hoc test, which compares all possible pairs.

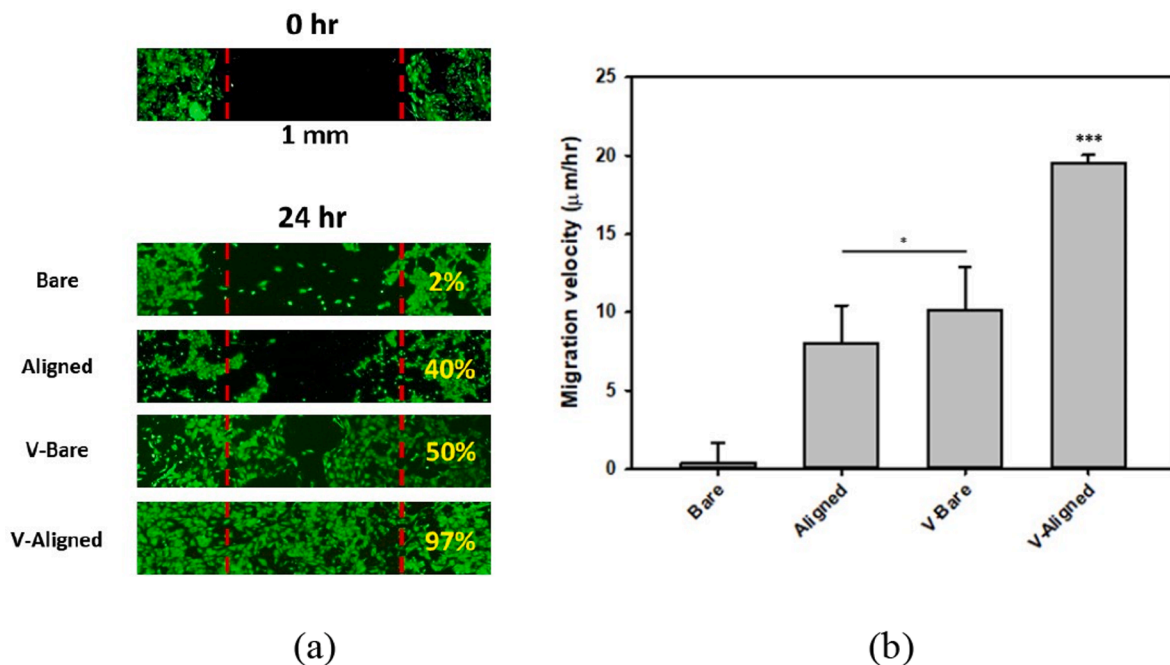


Fig. 8. Cell migration assay of HCAECs on different scaffold. (a) Fluorescence microscopy images visualizing HCAEC migration and resultant recovery ratio. (b) Migration velocity of HCAECs on different scaffold. The symbol *, **, and *** indicate statistical significance to a level of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, using one-way ANOVA with a Tukey post-hoc test, which compares with Bare group.

activated with extended pseudopodia and spreading on aligned scaffold (V-Aligned) without heparin. Activation of platelets was inhibited on the heparin-loaded scaffold (VH-Aligned), which showed a reduced

extension of pseudopodia in adhered platelets compared to the bare scaffold. These results confirmed that heparin release inhibits the adhesion and activation of platelets [43].

3.7. Biocompatibility *in vivo*

In vivo tissue biocompatibility of VH-Aligned PLLA was evaluated using a subcutaneous implantation mouse model. The extent of tissue response to implanted scaffold was investigated based on fibrotic tissue formation and inflammation score at the scaffold-host tissue interface. In the period after implantation, the weights of mice steadily increased and their body condition remained satisfactory. Inflammation scores in the operated tissues were zero for all experimental groups. The tissues around the scaffold were examined after H&E staining (Fig. 9). Less collagen fibrous capsules were formed at the surface-treated bottom side than the non-surface-treated top side, which is the bare PLLA side. These results indicate that HV-Aligned PLLA exhibit *in vivo* biocompatibility without inducing significant inflammation or collagen fibrous capsule formation.

4. Discussion

Topographical and biochemical cues were applied to facilitate the effective and rapid formation of endothelium on the surface of a PLLA-based blood contact material. Topographical cues were produced by aligned PLLA nanofibers and biochemical cues by immobilized VEGF.

Instead of using conventional printing techniques, such as photolithography, soft lithography, and nanoimprinting, which have the limitations of being complex, expensive, and applicable only to flat surfaces, we generated an aligned pattern on the PLLA substrate by thermally attaching aligned PLLA nanofibers. PLLA was chosen as the primary material component given its physical properties and proven biocompatibility; it has been successfully used in biodegradable stents and tissue engineering vascular grafts [44–46]. Glass temperature (T_g) of PLLA ranges from 50 to 80 °C and the melting temperature (T_m) is 180 °C. Therefore, heating PLLA to ~ 130 °C increases its viscosity and allows for the attachment of PLLA nanofibers (Fig. 2). The nanofibers were combined with the scaffold to form a single integrated PLLA-based

scaffold with aligned topography. Aligned nanofibers were firmly attached without delamination and maintained their aligned architecture. As only electrospinning and heating processes were used, generating aligned topography does not require expensive equipment or chemical treatment. The resultant PLLA scaffold is flexible and can be rolled into a tubular shape. Most of the blood-contact materials such as catheters, vascular grafts, and stents do not exist as two-dimensional flat and hard substrates. Instead, they have a tubular shape or a curved surface. Therefore, the flexibility of the scaffolds after thermal attachment is very important for the application to the final product of blood-contact materials.

VEGF was covalently immobilized to the fiber-attached PLLA scaffold via click chemistry to generate biochemical cues. VEGF is well known for enhancing endothelialization through a signaling cascade: upregulating ERK for cell proliferation, C-Src for cell migration, and Akt for cell survival in pro-apoptotic cellular microenvironments [27]. Surface modification of PLLA was necessary to bind functional groups for the covalent immobilization of VEGF. In this study, we performed aminolysis using HDA, a suitable method using diamine compounds to introduce amine groups onto polyester surfaces [47]. XPS and fluorescence analyses confirmed that HDA molecules were successfully grafted to PLLA via aminolysis and consequently, generated amine groups on the surface of PLLA scaffold. The amount of amine groups was dependent on aminolysis reaction time (Fig. 3). After amine groups were introduced to PLLA surface, VEGF was covalently immobilized on the PLLA surface via CuAAC, which is orthogonal click reaction between alkene and azide groups. In this study, alkene groups were attached to the PLLA scaffold and azide groups were conjugated to VEGF by using a heterofunctional NHS linker. We also tried CuAAC between alkene-conjugated VEGF and azide-incorporated PLLA scaffold, which turns out that much less amount of VEGF was immobilized than the other combination. This is due to possibility of alkynes to react with another alkyne independent of an azide group [48], which might cause the reactions between different alkene-conjugated VEGFs and the subsequent

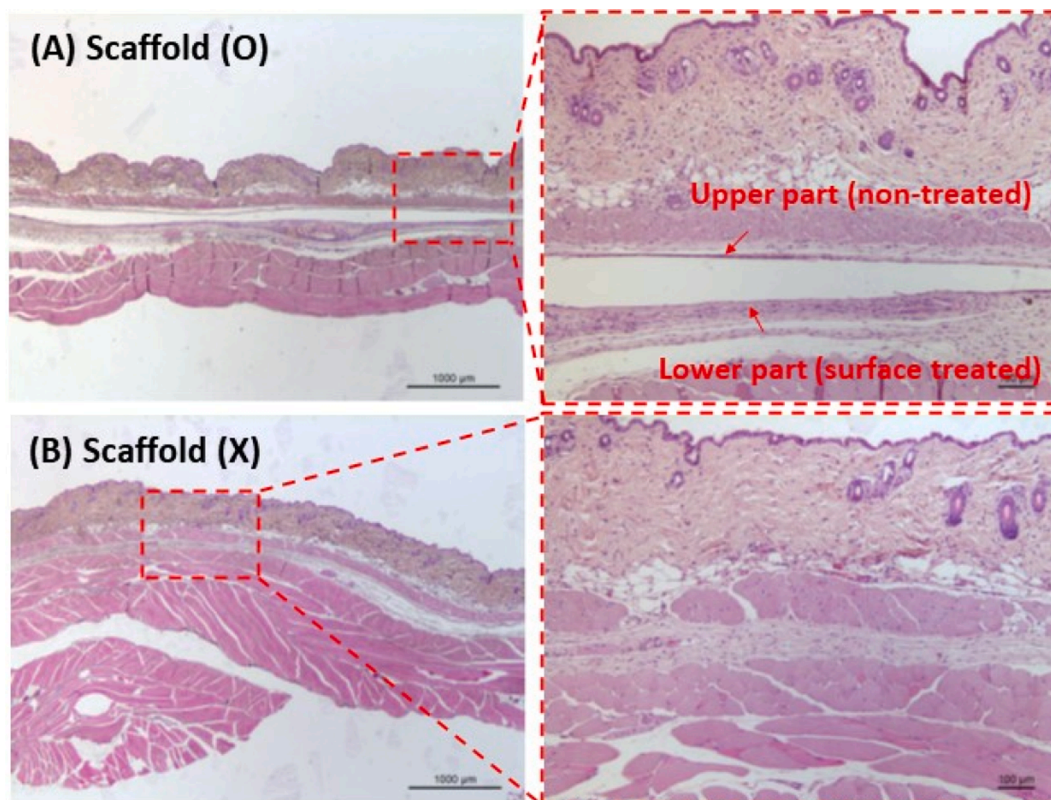


Fig. 9. Histopathological images of tissue with and without implanted VH/Aligned scaffold.

inefficiency of the click reaction between alkene-conjugated VEGF and the azide-incorporated PLLA scaffold.

Covalent immobilization of VEGF via CuAAC was confirmed with XPS, fluorescence and direct ELISA analysis (Fig. 4). VEGF immobilization through covalent bonding was much more effective than physical adsorption and demonstrates the effectiveness of the CuAAC method. With more amine groups being generated on PLLA with increased aminolysis reaction time, more VEGF was immobilized onto scaffolds that underwent longer aminolysis. In addition, more VEGF was immobilized onto fiber-attached than to flat PLLA scaffold, indicating that the fibers were not only responsible for providing topographical cues but also for increasing the surface area for VEGF immobilization.

After topographical and biochemical cues were successfully incorporated into the PLLA scaffold, their effects on endothelialization were examined at the *in vitro* level based on: the proliferation of HCAECs, expression of an endothelial marker CD31, and migration of HCAECs. Based on the MTT assay (Fig. 6a), the degree of proliferation revealed that topographical cues had a positive effect on initial cell adhesion due to increased surface area. However, they had little effect on long-term cell proliferation. It was reported that early cell adhesion is strongly dependent on the roughness of substrates. Generally, nano- or sub-micrometer scale topographical features promoted cell adhesion by providing more extensive surface area and through the formation of more focal adhesion between cells and substrates [49–51]. Therefore, Aligned and V(S)-Aligned groups showed higher absorbance values than V-Bare groups at Day 1 due to attached nanofibers which could provide more surface area and topographical cues to cells. However, cell proliferation of V-Bare group is much higher than those of Aligned and V(S)-Aligned groups in Day 4 and Day 7 because biochemical cues (VEGF) play a more important role in long-term cell culture. Significant proliferation observed with the VEGF-immobilized PLLA scaffolds resulted from the metabolic activities of HCAECs being inhibited in the absence of VEGF, a signaling molecule in the ERK pathway [30,52]. Surface-bound VEGFs proved more effective in cell proliferation than the same amount of soluble VEGF (Fig. S3a in Supporting Information); surface-bound VEGFs have resistance to clearance by endocytosis, which allows for a prolonged effect and multivalency [27,53]. The aligned topography also induced the elongation of HCAECs parallel to the aligned fibers (Fig. S4 in Supporting Information). As endothelial cells are naturally aligned along the direction of blood flow [54], the aligned topography on the PLLA scaffold simulates morphology more similar to what is observed *in vivo*. Similar to cell proliferation, the expression of CD31 was significantly upregulated only on the VEGF-immobilized PLLA scaffold and there was no distinct effect of aligned patterns on the expression of CD31 without surface-bound VEGF (Fig. 7). Higher expression of CD31 was observed with scaffolds having aligned architecture than flat scaffolds, likely because of greater amount of immobilized VEGF on the fiber-attached PLLA scaffold (Fig. 4). As relatively increased expression of CD31 represents the formation of a mature endothelium with stable cellular interaction [55,56], this indicates that immobilized VEGF enhanced re-endothelialization on the PLLA scaffolds by promoting endothelium maturity. Unlike the results for cell proliferation and CD31 expression, both surface-bound VEGF and aligned architecture improved HCAEC migration (Fig. 8). We, therefore, suggest that HCAEC migration was promoted through signaling cascades initiated by VEGF and anisotropic aligned topography directing cell movements [57,58]. The *in vitro* experiments demonstrated that increased cell proliferation and CD31 expression were mainly promoted by surface-immobilized VEGF, whereas cell migration was promoted by a combination of VEGF and aligned nanofibers.

A key feature of the proposed PLLA-based scaffold is that thermally attached nanofibers not only provide an aligned topography but also serve active functions, such as drug delivery. This is possible due to the independent preparation of the nanofibers where the physical and chemical properties can be determined before assembly with the scaffold, regardless of the substrate material. Heparin was chosen as model

drug in this study as it promotes the blood compatibility of biomaterials. In addition, heparin inhibits SMC proliferation by repressing immediate-early genes, matrix-degrading protease, and mitogen-activated protein kinase [59]. It could therefore prevent neointimal hyperplasia, which is currently a major clinical problem in blood contact devices. Heparin-loaded nanofibers were attached to the PLLA substrate and underwent the same process of VEGF immobilization. The generated scaffold could sustain heparin release for one month. Heparin had no adverse effect on surface endothelialization but inhibited the proliferation of HCASMCs (Fig. S6 in Supporting Information). Finally, we evaluated the effect of heparin on blood compatibility through the adhesion and activation of platelets on the different PLLA-based scaffolds. The combination of heparin and VEGF showed the most promising results in terms of lower platelet adhesion and activation, which was due to the anti-coagulating effect of heparin and the increased hydrophilicity of immobilized VEGF (Fig. S7 in Supporting Information). These results emphasize the potential of the proposed scaffold to minimize thrombosis and neointimal hyperplasia.

The *in vivo* biocompatibility experiments revealed that HV-Aligned PLLA showed similar levels of biocompatibility to bare PLLA, which confirms that the physical and chemical surface treatments used in this study had no negative effect on the biocompatibility of the scaffold (Fig. 9).

5. Conclusion

Topographical and biochemical surface modifications promoted endothelialization on a PLLA scaffold and can potentially be applied in the research and development of blood-contact materials. Aligned architecture was prepared by thermal-attachment of electrospun nanofibers, whereas VEGF was covalently immobilized onto the PLLA scaffold via CuAAC-mediated click chemistry. The resultant PLLA scaffold maintained both topographical and biochemical cues without delamination of attached nanofiber and leaching of VEGF. The scaffold with both surface-immobilized VEGF and topographical guidance synergistically promoted proliferation, CD31 expression, and HCAEC migration. As the aligned nanofibers were prepared separately and assembled onto the main PLLA substrate, additional active functions such as drug delivery could be incorporated. Heparin-loaded PLLA scaffolds achieved sustained release of heparin, which suppressed the attachment and activation of platelets as well as the proliferation of HCASMCs without affecting surface endothelialization. The scaffold was also biocompatible *in vivo*. In summary, we proposed simple and orthogonal surface modification techniques that can enhance biocompatibility, endothelialization, anti-thrombosis, and anti-restenosis in vascular intervention therapies.

CRedit authorship contribution statement

Seungmin Kang: Conceptualization, Formal analysis, Methodology, Investigation, Data curation, Writing – original draft. **Yu Jin Chi:** Investigation. **Kanghee Cho:** Conceptualization, Formal analysis, Investigation. **Hyun Jong Lee:** Investigation, Supervision, Writing – review & editing. **Won-Gun Koh:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsusc.2022.152969>.

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