Biomaterials 31 (2010) 9431–9437

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Human endothelial cell growth on mussel-inspired nanofiber scaffold for vascular tissue engineering

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ARTICLE INFO

Article history:
Received 3 August 2010
Accepted 27 August 2010
Available online 28 September 2010

Keywords:
Endothelial cells
Nanofibers
Electrospinning
Mussel adhesives
Poly(dopamine)
Vascular tissue scaffold

ABSTRACT

The endothelialization of prosthetic scaffolds is considered to be an effective strategy to improve the effectiveness of small-diameter vascular grafts. We report the development of a nanofibrous scaffold that has a polymeric core and a shell mimicking mussel adhesive for enhanced attachment, proliferation, and phenotypic maintenance of human endothelial cells. Polycaprolactone (PCL) was chosen as a core material because of its good biodegradability and mechanical properties suitable for tissue engineering. PCL was electrospun into nanofibers with a diameter of approximately 700 nm and then coated with poly (dopamine) (PDA) to functionalize the surface of PCL nanofibers with numerous catechol moieties similar to mussel adhesives in nature. The formation of a PDA ad-layer was analyzed using multiple techniques, including scanning electron microscopy, Raman spectroscopy, and water contact angle measurements. When PDA-coated PCL nanofibers were compared to unmodified and gelatin-coated nanofibers, human umbilical vein endothelial cells (HUVECs) exhibited highly enhanced adhesion and viability, increased stress fiber formation, and positive expression of endothelial cell markers (e.g., PECAM-1 and vWF).

1. Introduction

Attempts to develop a small-diameter vascular graft (<6 mm) have been challenging over the past decade due to thrombosis and intimal hyperplasia [1]. The seeding of endothelial cells onto the luminal surface of the graft is considered to be a potential route to improving the patency of small-diameter prostheses [2]. Since endothelial cells release factors that regulate thrombogenesis/fibrinolysis and platelet activation/inhibition [3], the endothelialization of a graft surface effectively renders the surface to become antithrombogenic [4]. Since the attachment of endothelial cells occurs on the surface of an extracellular matrix (ECM) in nature, the fabrication of biomaterials analogous to ECM could aid the attachment, proliferation, and phenotypic maintenance of endothelial cells on vascular scaffolds.

Recently, electrospinning technology has been intensively studied as a method to create ECM-mimicking structures [5–10]. Electrospun fibers have a structural similarity to natural ECM components as well as many advantages during processing; they can be produced on a large scale, and their diameters can be readily controlled to make them nanometer-to-micrometer sizes [5]. Synthetic polymers, such as polycaprolactone [6,7], poly(lactide-co-glycolide) [8,9], and poly(lactide-co-caprolactone) [10], have been electrospun into nanofibers, which exhibit excellent mechanical properties [11]. In order to utilize electrospun fibers as a tissue engineering scaffold, however, surface modification is critical since synthetic polymers often suffer from very poor cell adhesion due to their hydrophobicity and the lack of cell recognition sites [7,10].

To date, numerous strategies of surface modification have been reported to allow for better attachment of cells, including plasma treatment, physical adsorption, and chemical immobilization of ECM molecules or cell recognition peptides [12,13]. However, these methods have drawbacks, such as the complicated steps required for chemical immobilization, the limited penetration depth of plasma, limited applicability to substrate materials, etc. Recently, we have developed a new surface modification method for cell adhesion on non-wetting surfaces, inspired from the adhesion mechanism of mussels [14]. These mollusks can attach to any type of materials, including organic and inorganic substances, and this universal adhesive property relies on the repeated 3,4-dihydroxy-L-phenylalanine-lysine (DOPA-K) motif in mussel foot proteins [15,16]. Inspired by this motif, researchers have used small molecules containing catecholamine moiety, such as dopamine, as an adhesive agent that mimics the mussel's adhesion [17].

In this study, we functionalized the surface of electrospun polycaprolactone (PCL) nanofibers with catecholamine ad-layer to enhance the attachment and proliferation of endothelial cells as

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doi:10.1016/j.biomaterials.2010.08.071

a potential application in vascular tissue engineering. PCL was electrospun into nanofibers and then treated with dopamine, which spontaneously polymerizes to poly(dopamine) (PDA) in an aqueous solution. The formation of the PDA shell on electrospun PCL nanofiber core was studied using scanning electron microscopy, Raman spectroscopy, and water contact angle measurements. The attachment and viability of human umbilical vein endothelial cells (HUVECs) on PDA-coated nanofibers were evaluated and compared with those of unmodified and gelatin-coated nanofibers. We further analyzed the cytoskeleton development and the expression of endothelial cell markers, such as platelet endothelial cell adhesion molecule (PECAM-1) and von Willebrand factor (vWF).

2. Materials and methods

2.1. Materials

Polycaprolactone (Mn = 80,000), dopamine hydrochloride, and gelatin were purchased from Sigma-aldrich (MO, USA). Chloroform, dimethylformamide (DMF), and dimethylsulfoxide (DMSO) were obtained from Junsei (Japan), Merck (Germany), and Junsei (Japan), respectively, and were used without further purification. Various substrates were provided from the following manufacturers: glass, Marlenfeld GmbH (Germany); poly(dimethylsiloxane), Dow Corning (MI, USA); silicone rubber, Hanmi Rubber & Plastics (Korea); poly(tetrafluoroethylene), Hanmi Rubber & Plastics (Korea); and polyethylene, CS Hyde Company (IL, USA).

2.2. Preparation of PCL nanofibers and surface modification

PCL nanofibers were fabricated by employing an electrospinning process using a rotating collector (NanoNC, Korea). After dissolving PCL in chloroform/DMF (2:1 v/v) at a concentration of 20 wt.%, the electrospinning process was carried out with the following parameters: applied voltage, 25 kV; solution feed rate, 10 μl/min; distance between needle and collector, 15 cm; syringe needle gauge, 21 G; spinning time, 60 min. The surface modification with poly(dopamine) was performed by a simple immersion of electrospun PCL nanofibers into a dopamine solution (2 mg/ml in 10 mM Tris, pH 8.5) at 25 °C for 16 h. The modified substrates were washed with water and then dried with nitrogen gas. For gelatin coating, nanofibers were dipped in a gelatin solution (0.2 wt.% in PBS, pH 7.4) at 37 °C for 16 h. The resulting nanofibers were observed using scanning electron microscopy (Hitachi S-4800, Japan), and surface modifications were characterized by using Raman spectroscopy and water contact angle measurements. Raman spectra were obtained by accumulating 50 scans with a resolution of 2 cm^{-1} in the range of 1000–1800 cm^{-1} using the LabRAM HR UV/Vis/NIR (Horiba Jobin Yvon, France). The water contact angle was determined by using a contact angle analyzer (Surface Electro Optics Co., Korea).

2.3. Cell culture and viability assay

HUVECs were maintained in endothelial basal medium-2 (EBM-2) with supplements (hEGF, hydrocortisone, GA-1000, FBS, VEGF, hFGF-B, R^3-IGF-1, ascorbic acid, and heparin; Lonza, Switzerland). Cells were subcultured at least twice a week and were maintained at a humidified atmosphere of 95% air and 5% CO_2. HUVECs below passage 20 were used for this study. Before cell seeding, the unmodified, gelatin-coated, and PDA-coated PCL nanofibers were sterilized using a 70% ethanol solution. HUVECs were placed in a 24-well plate and then incubated for 48 h at 37 °C. The cell density was 5×10^4 cells/ml for PCL nanofibrous substrates or 3×10^4 cells/ml for various 2-dimensional substrates. The viability of HUVECs on the unmodified, gelatin-coated, and PDA-coated PCL nanofibers was evaluated using Live/Dead cell assay (Invitrogen, CA, USA) and MTT assay. The Live/Dead cell assay was carried out following the manufacturer's instructions, and the cells were observed using a laser scanning confocal microscope (LSCM; LSM510, Carl Zeiss, Germany). We carried out two independent experiments with duplicate samples, and the number of live cells was counted at ten independent sites of each sample. The MTT assay was performed by following these steps: addition of 100 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution (5 mg/ml in PBS), incubation at 37 °C for 3 h, removal of media, dissolution of purple formazan using DMSO, and measurement of absorbance at 595 nm. We performed two independent experiments with triplicates.

2.4. Cytoskeleton organization and phenotype study

The cytoskeleton organization of HUVECs grown on unmodified, gelatin-coated, and PDA-coated PCL nanofibers was analyzed by using actin staining. After 48 h cultivation, cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, stained with Rhodamine-phalloidin (Invitrogen, CA, USA) for 20 min, and stained with Hoechst (Sigma-aldrich) for 10 min under dark conditions. The stained actin filaments were observed using LSCM. The expression patterns of platelet endothelial cell adhesion molecule (PECAM-1) and von Willebrand factor (vWF) of HUVECs were analyzed by immunocytochemistry. After 48 h cultivation, cells were fixed with 4% paraformaldehyde for 15 min, made permeable with 0.1% Triton X-100 for 10 min, blocked with 1% bovine serum albumin for 30 min, treated with a primary antibody for overnight at 4 °C, incubated with a secondary antibody for 1 h at room temperature, and then stained with 500 nM propidium iodide for 5 min. Antibodies were used at the following concentrations: rabbit polyclonal anti-human PECAM-1 (ab28364; Abcam, UK), 1:100; rabbit polyclonal anti-human vWF (ab6994; Abcam, UK), 1:50; Alexa488-conjugated goat anti-rabbit IgG, 1:500 (Invitrogen, CA, USA). The stained samples were visually observed using LSCM.

2.5. Statistical analysis

All the quantitative results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out by means of one-way analysis of variance (ANOVA). A p-value less than or equal to 0.05 was considered statistically significant.

3. Results and discussion

In order to introduce cell attachment moieties to hydrophobic nanofibers made of synthetic polymers, we utilized mussel-inspired poly(dopamine) (PDA) as a surface modification material. As depicted in Fig. 1A, we hypothesized that PDA can cover the surface of 3-dimensional nanofiber mesh as a shell and enhance the adhesion of endothelial cells. For the proof-of-concept, polycaprolactone (PCL) nanofibers were fabricated using an electrospinning process and then modified with PDA by immersing the nanofibers into an aqueous dopamine solution. PCL was chosen as a model scaffold material because of its good biodegradability and mechanical properties suitable for tissue engineering [6,7]. For comparison, PCL nanofibers were also coated with gelatin, which has been widely used for enhancing cell adhesion [18,19]. The morphology and diameter of modified nanofibers were analyzed using SEM (Fig. 1B); non-woven PCL nanofibers were randomly oriented and presented a porous network that enables cell infiltration and efficient mass transfer of nutrients and gases, which is desirable in ECM-mimicking materials. The diameter of unmodified, gelatin-coated, and PDA-coated PCL nanofibers was 720.8 ± 310.5 nm, 726.5 ± 356.8 nm, and 760.4 ± 325.1 nm, respectively. It was difficult to confirm the PDA formation solely by the increased diameter of PDA-coated PCL nanofibers because the diameter difference between unmodified and modified nanofibers was not statistically significant. It is noteworthy that the thickness of PDA layer is approximately 40 nm after 16 h incubation according to a previous report [17]. We further confirmed the PDA coating by using Raman spectroscopy and water contact angle measurement. In Raman spectra (Fig. 1C), new peaks at 1345 and 1603 cm^{-1}, which correspond to the aromatic component of PDA [20], appeared in addition to the characteristic peaks of PCL, such as $\nu C=O$ at 1727 cm^{-1}; δCH_2 at 1421, 1444, 1469 cm^{-1}; ωCH_2 at 1287, 1308 cm^{-1}; and skeletal stretching at 1112 cm^{-1} [21]. We measured water contact angles of unmodified and modified PCL NFs (Fig. 1D). Unmodified PCL NFs showed the higher static contact angle (92.4 ± 5.2°) than that of homogenous PCL films (83.0°), possibly due to the air trapped within pores of fibrous meshes [18]. The contact angle hysteresis is ascribed to the surface roughness of nanofiber mats (Fig. S1 in Supporting information). After the gelatin or PDA coating, we observed drastic decreases in the water contact angle to 0.0°, which indicates that each coating changed the PCL property from hydrophobic to hydrophilic one. After the surface modification with gelatin or PDA, water drop was immediately absorbed into fibrous networks, resulting in a zero contact angle.

The effect of PDA coating on cell adhesion and viability was investigated by culturing human umbilical vein endothelial cells (HUVECs) on unmodified and modified PCL nanofibers. As shown in Fig. 2A, the morphology and the number of attached HUVECs were dependant on the type of surface modification. The number of live cells also increased on PDA-coated PCL nanofibers in comparison to