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Preparation and characterization of spiral-like micro-struts with nano-roughened surface for enhancing the proliferation and differentiation of preosteoblasts

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ABSTRACT

Factors that are important for the development of tissue-engineered scaffolds include an appropriate selection of the physiochemical structure and surface characteristics to achieve the desired cellular responses depending on the target tissue or organ. In this study, we designed the linear polycaprolactone (PCL) strut, a spiral-like PCL strut that was fabricated using a 3D melt-printing system and a modified spiral-like PCL strut via a 3D melt-printing/plasma-etching process. The surface-roughened spiral-like strut showed significantly enhanced wettability and protein absorption abilities, which were closely related to cellular activities compared to those of linear and spiral-like struts. The *in vitro* cellular activities using the preosteoblasts (MC3T3-E1) indicated that the newly designed surface-modified spiral-like structure showed significantly higher metabolic activities and mineralization compared to those of conventionally 3D-printed struts.
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Introduction

Tissue engineering is an interdisciplinary science that requires the understanding of many different fields such as material science, cell biology, and mechanical engineering for the replacement and restoration of tissues and organs [1,2]. As such, tissue-engineered scaffolds play an important role as a 3D construct that must possess *in vivo*-like mechanical properties to enhance cellular activities and provide sufficient space for tissue formation [3]. Therefore, scaffold properties are mainly determined by the selection of biomaterial.

One key factor is surface topology, which affects cell behaviors such as the initial cell attachment, proliferation, and differentiation. In particular, anchorage-dependent cells interact with substrate surfaces through integrins, one type of transmembrane adhesion receptors, by transducing signals to the cell cytoskeleton [4,5]. The bidirectional signaling pathway between the substrate surface and the cytoskeleton of the cells can regulate cellular activities such as cell growth, differentiation, and apoptosis [6–9].

In general, biomaterials can be categorized into two groups: natural and synthetic polymers. Natural polymers, such as collagen, chitosan, gelatin, silk fibroin, cellulose, starch, and alginate, are derived from natural sources [10]. Another type of natural biomaterial is extracellular matrix (ECM) derived from the targeted tissue. For bone tissue regeneration, La et al. used bone demineralized and decellularized ECM solution for stimulating bone formation [11]. Generally, naturally derived polymers show outstanding hydrophilicity and biocompatibility. However, these polymers are difficult to process, which limits their use in biomedical scaffolds. On the contrary, synthetic polymers, such as poly(vinyl alcohol), poly(lactic-co-glycolic acid), poly(glycolic acid), poly(L-lactic acid), and polycaprolactone (PCL), are relatively easy to process, have mechanical and degradable properties that can be manipulated, and have relatively non-toxic and non-immunogenic properties; however, these materials lack biocompatibility [12].

PCL is a synthetic bioresorbable polymer that has been commonly used in the regeneration of bone and cartilage because of its outstanding mechanical properties, slow degradation, and ease of forming 3D porous shapes [13]. However, the hydrophobic characteristic and smooth surface morphology after various processes of PCL presents a crucial disadvantage for it to be used as a material in tissue engineering. Therefore, to improve the

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functionalization of PCL, various surface modification techniques have been applied. To overcome these problems, various surface modifications using chemical, physical, and biological methods have been applied because surface topography of the material can directly interact with the seeded cells [14,15]. Oyane et al. treated the PCL surface with NaOH to form a bone-like apatite layer [16]. Tiaw et al. modified a PCL surface by producing holes on the surface using femtosecond and excimer laser [17]. Zhang et al. successfully immobilized RGDC peptides on a PCL scaffold surface to improve cell-substrate interactions [18].

Another simple method to modify surface topology is by using a plasma process [19]. Plasma treatment positively influences the surface roughness, wettability, compositions, and hydrophilicity without changing the bulk properties of the plasma-treated scaffold [20]. Another advantage is that this technique does not use toxic solvents, which can be harmful to the viable cells. In general, although the plasma process is applied to polymer surfaces to enhance hydrophilicity, recent nanoscale roughened surfaces on the scaffold have been obtained using a selective plasma etching process [21].

In our previous study, we developed a PCL scaffold with a 3D mesh structure comprising spiral-like struts, which showed significantly improved cellular responses such as cell proliferation and osteogenic differentiation [21]. This phenomenon may be due to the inhomogeneous pore sizes of the scaffold, which can induce dynamic cell–cell interactions, therefore affecting the osteogenic differentiation. Although a unique pore structure consisting of spiral-like struts having some wave-angle can provide significant improvements in cellular activities, we believe that the spiral-strut itself can also affect cellular activities due to the great surface-to-area ratio of the spiral-like morphology, which contributes cell attachment and proliferation [22]. Based on our previous studies, we have tried to modify the surface of the spiral-like strut to improve the cell adhesion and proliferation on the strut by using a plasma etching process.

By using the selected condition of the plasma etching process, we fabricated a spiral-like PCL strut with chemically and physically modified surfaces, and then we evaluated the effects of the chemical and physical topography of the spiral-like struts on the cellular activities of the preosteoblasts. For the evaluation of physical and biological properties, three types of struts (linear PCL [LP], spiral-shaped PCL [SP], and nano-roughened spiral PCL [RSP] struts) were used. We studied the processability of the spiral-like struts and performed physical and topological tests such as wettability, protein absorption, mechanical strength, and surface roughness. For the *in vitro* tests, preosteoblasts (MC3T3-E1 cells) were seeded onto the struts and tests were performed to assess cell proliferation and osteogenic differentiation.

The goal of this study is to evaluate the physical and biological properties of the basic unit of the scaffold, which is the strut. Therefore, we used surface modified spiral-like struts to obtain synergetic effect between spiral-like shape and nano-roughened surface for enhanced cellular activities.

Materials and methods

Materials

The struts were fabricated using PCL (M_n = 45,000 g mol^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) for melt-printing.

Scaffold fabrication

Fabrication of PCL, PS, and PSP struts

The struts were fabricated using a melt-printing system employed by an automated 3D robot system. To print the struts onto the working stage, PCL pellets were heated in a cylindrical heating barrel at 110 °C and then extruded through a 250-μm nozzle at a constant pressure of 450 ± 10 kPa.

Surface roughness by plasma treatment

The surfaces of the melt-printed struts were modified using an oxygen plasma system (CUTE-MP/R; Femto-Science, Inc., Gyeonggi, South Korea). A low frequency (LF) source operating at 50 kHz, power of 20 W, pressure of 5.41 × 10^{-1} Torr, and gas flow rate of 10 standard cubic cm per min (sccm) was applied 3 times for 1 h each.

Characterization of the scaffolds

The strut and surface morphology were analyzed using an optical microscope (BX FM-32; Olympus, Japan) and scanning electron microscopy (SEM: SNE–3000 M, SEC Inc., South Korea).

The surface topographies of the untreated and plasma-treated struts were evaluated using a Laser Scanning Microscope (LSM: VK-9710, Keyence, Japan). We determined the average roughness (R_a) and root mean square roughness (R_{rms}) for PCL, PS, and PSP struts.

The mechanical properties were determined using a tensile machine (Toptech 2000; Chemilab, South Korea). Struts were stretched at a speed of 0.5 mm s^{-1}.

X-ray photoelectron spectroscopy (XPS) measurements were performed using a hemispherical electrostatic energy analyzer and an Al Kα (1486.6 eV) X-ray source. XPS spectra were measured in 0.05 eV steps.

The hydrophilicity of the struts was evaluated by measuring the static water contact angle using a droplet of water (10 μL), which was placed on the surface of 3 struts.

For measuring the protein absorption, we placed the struts in αMEM-containing 24-well plates and incubated them for 1, 6, or 12 h at 37 °C. After rinsing with PBS, the struts were treated with 0.1% Triton X-100. An aliquot of the lysate (25 μL) and bicinchoninic acid (BCA) protein assay (Pierce Kit; Thermo Scientific, MA, USA) working reagent (200 μL) were added and then incubated for 30 min at 37 °C. The absorbance of the mixture was measured at 562 nm in a spectrophotometer (EL800; Bio-Tek Instruments, Winooski, VT, USA).

For the evaluation of biodegradability, the samples were immersed in PBS for 14 days under incubation circumstances. The degradation rate was calculated by the following equation: degradation rate (%) = $[(W_i - W_t)/W_i] \times 100$, where W_i is the weight before degradation and W_t is the weight after degradation.

MC3T3-E1 cell culture

Mouse preosteoblast cells (MC3T3-E1; ATCC, Manassas, VA, USA) were used. The cells were cultured in alpha-Minimum Essential Medium (α-MEM) (Life Sciences Advanced Technologies Inc., St Petersburg, FL, USA) containing 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA, USA) and 1% antibiotic-antimycotic (Cellgro, Herndon, VA, USA).

Cell seeding

Prior to the *in vitro* tests, the struts were sterilized using 70% ethanol and ultraviolet (UV) light. A 30 μL cell suspension containing 1 × 10^5 cells was then seeded onto the struts and incubated at 37 °C with 5% CO_2 for 1, 3, 7, and 14 days.

Cell proliferation

The MTT assay, a tetrazolium-based colorimetric assay (MTT assay: Cell Proliferation Kit I; Boehringer Mannheim, Mannheim,

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