Effect of oxygen plasma etching on pore size-controlled 3D polycaprolactone scaffolds for enhancing the early new bone formation in rabbit calvaria

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This study was to investigate the effects of O₂ plasma-etching of the 3D polycaprolactone (PCL) scaffold surface on preosteoblast cell proliferation and differentiation, and early new bone formation. The PCL scaffolds were fabricated by 3D printing technique. After O₂ plasma treatment, surface characterizations were examined by scanning electron microscopy, atomic force microscopy, and contact angle. MTT assay was used to determine cell proliferation. To investigate the early new bone formation, rabbits were sacrificed at 2 weeks for histological analyses. As the O₂ plasma etching time is increased, roughness and hydrophilicity of the PCL scaffold surface increased. The cell proliferation and differentiation on plasma-etched samples was significantly increased than on untreated samples. At 2 weeks, early new bone formation in O₂ plasma-etched PCL scaffolds was the higher than that of untreated scaffolds. The O₂ plasma-etched PCL scaffolds showed increased preosteoblast differentiation as well as increased new bone formation.

Keywords: 3D printing, Polycaprolactone, Oxygen plasma etching, Scaffolds, New bone formation

INTRODUCTION

The need for repair of bone defects has been increasing because of tumor ablative surgery, congenital defects, fractures, oral and maxillofacial treatment, osteoporosis and other causes of loss of skeletal tissue ¹⁻⁴. The defect sizes, the quality of the soft tissue covering the defect, and the reconstructive method have a significant effect on the clinical success ⁵. Traditional surgical techniques used in reconstruction have been used to autograft and allograft transplantations; however, their disadvantages involve donor site morbidity, limited supply, and risk of significant resorption⁶.

In order to overcome these problems, synthetic bone substitutes have attracted attention as promising alternative to replace part of the bone defect. Scaffolds serve as matrices for tissue formation, and surface properties promoting cell adhesion, proliferation, and differentiation, as well as desirable mechanical strength and osteoconductivity, are all essential⁷.

Recently, there is a great interest in the development of new processes to fabricate three-dimensional (3D) scaffolds that interact favorably with cells. The conventional techniques for 3D scaffold fabrication, including salt leaching, gas foaming, solvent casting, melt molding, and electrospinning have some limitations⁸⁻¹³. These technologies are unable to provide precise control over the pore size, pore geometry, pore interconnectivity, nor the construction of internal channels within the scaffold.

In this work, rapid prototyping (RP) technologies were used to solve these problems. Controlled 3D structures can be fabricated through RP techniques, also known as solid freeform fabrication (SFF) technology. Among the SFF techniques, the fused deposition modeling (FDM) method does not require any solvent and offers great ease and flexibility in material handling and processing. FDM uses a temperature controlled extruder to pull out a thermoplastic material and deposit the molten polymer onto a platform in a layer-by-layer process. In addition, FDM is highly reproducible, with a relatively moderate speed, which enables control over the major physical characteristics of the resulting scaffold, such as mechanical properties, porosity, and pore shape¹⁶.

In extrusion 3D printing, a biomaterial (e.g., polycaprolactone) can be melted and extruded in a computer-controlled pattern to construct scaffolds, laying down layer on top of layer to create patient-specific customized scaffolds¹⁵. Poly-ε-caprolactone (PCL) is bioresorbable and nontoxic polymer that has been investigated alone or in combination, for bone applications, as have osteoconductive materials such as tricalcium phosphate, hydroxyl apatite, and calcium phosphate¹⁶⁻²².

Various studies have investigated the effect of scaffold architectures bone growth, with many hypothesizing that results are dependent on the fluid flow and nutrient/waste diffusion properties imposed by the design parameters utilized²³⁻²⁵. Roosa et al determined that different pore sizes (350, 500, and 800 µm) had little effect on in vivo bone growth in PCL scaffolds²⁶. Others have concluded that scaffold porosity is important for cell delivery and sufficient diffusion of nutrients and waste into and out of the scaffold²⁶. Many studies support or refute the requirement of specific pore sizes and shapes, strut/fiber diameters, interconnectivity, or porosities for optimal bone growth²⁷.
In order to enhance the hydrophilic and bioactive properties of PCL scaffolds, various surface modification techniques have been developed, including chemical treatments, blending, laser surface modification, ion beam radiation, plasma modification, protein coating or immobilization and peptide grafting. Among them, the plasma surface modification technique has the advantage of modifying the surface properties without changing the intrinsic bulk properties of the PCL substrates. Plasma modification also has the advantage of being free of residual solvents on the substrate surface in contrast to wet chemical surface modification techniques. These results demonstrate the feasibility of plasma technology to modulate the biological functions of scaffolds.

However, the effects of physicochemical surface properties and different pore sizes of 3D PCL scaffolds, on early new bone formation in vivo, are yet to be clarified.

Herein, we hypothesize that the combination of scaffold designs (different pore size and lay-down pattern) and scaffold surface characteristics (nano/micro surface roughness and surface chemistry) might have a synergistic effect on preosteoblast cell proliferation, differentiation, and early new bone formation. Thus we have performed in vitro and in vivo experiments to find the important variables that affect the 3D PCL scaffold performance.

MATERIALS AND METHODS

Fabrication of 3D PCL scaffolds

PCL (average molecular weight; 45,000, Sigma-Aldrich, St. Louis, MO, USA) was used as the material for the scaffold. The 3D PCL scaffold was fabricated using Bio-Extruder equipment (3D Bio Printer, M4T-100, M4T, Daegu, Korea). The 3D scaffolds architecture was designed using a computer-aided design (CAD) system, which can produce a well-defined internal and external shape with different pore sizes for cell ingrowth. The PCL pellets were melted at 90°C in a heating cylinder. Molten PCL was extruded through a nozzle by compressed dry air at 580 kPa pressure. The inner diameter of the nozzle was 300 µm, and the PCL strut could be deposited layer-by-layer at a feed rate of 200 mm/min. Finally, the 3D PCL scaffold was fabricated into a disc shape with a diameter of 8 mm and height of 1.8 mm.

To investigate the contact angles before and after O₂ plasma etching, PCL film was prepared using a flat glass plate. Briefly, PCL pellets were melted by a hot plate at 60°C, poured into another glass plate, and subsequently pressed by another glass plate.

The internal architectures of the 3D PCL scaffolds were constructed with a specific lay-down pattern (0°/45° or 0°/90° shift) and pore size (150, 250, or 350 µm). The scaffolds were designed with three different pore sizes and two lay-down patterns and are denoted as 45/150, 45/250, 45/350, 90/150, 90/250, and 90/350 3D PCL scaffolds, respectively.

Oxygen plasma etching on 3D PCL scaffolds

The 3D PCL scaffolds were treated with oxygen plasma under anisotropic etching conditions to improve the nano and micro surface roughness. The O₂ plasma etching was performed by radio frequency (RF, 13.56 MHz) capacitively-coupled plasma (Miniplasma Station, Daejeon, Korea). Reactive ion etching (RIE) was applied to achieve the anisotropic profile introducing RF discharge power to a bottom electrode. The samples were mounted on an upper stage in the vacuum chamber. The oxygen plasma etching process was performed under the following conditions: the RF discharge power was set at 100 W, oxygen gas flow rate at 20 sccm, and working pressure under 100 mTorr. To make a different surface roughness on the 3D PCL scaffolds surface, the surface was etched for 1 and 24 min. During the O₂ plasma etching process, etching was performed for 3 min and stopped for 3 min alternately, to minimize the thermal damage; this process was repeatedly 8 times. In addition, in order to perform the homogeneous O₂ plasma etching to the interior structure of 3D PCL scaffold, we carried out O₂ plasma etching the upper surface of the 3D PCL scaffold, and then turned it over again to perform O₂ plasma etching on the lower surface.

Surface analysis before and after oxygen plasma etching

Water contact angles were used to evaluate surface wettability by measuring the degree of water spreading on the PCL film surface. The hydrophilicity of samples was examined by the sessile drop method using a goniometer (GS, Surface Tech, Gwangju, Korea). A water droplet (5 µL) was dropped by a syringe mounted vertically against the PCL films surface. After the water was applied on the surface for 5 s, the droplet arc and the angle of contact (θ) at the interface were measured and recorded by a charge-coupled device (CCD) camera.

The change in surface morphology before and after O₂ plasma etching was observed with a scanning electron microscopy (SEM, SNE-3200M, SEC, Suwon, Korea) and field emission SEM (FE-SEM, S-4800, Hitachi, Tokyo, Japan). All samples were pre-coated with a conductive layer of sputtered gold. The micrographs were taken at an accelerating voltage of 5 or 10 keV at magnifications of 50× or 5,000×.

The surface topology and roughness of the 3D PCL scaffolds were analyzed by atomic force microscopy (AFM, XE-100, Park systems, Suwon, Korea) under non-contact mode with a scan rate of 0.1 Hz. The scan area was 5×5 and 10×10 µm, randomly selected from the scaffold surface. An arithmetic mean of root mean square roughness (Rq) was calculated directly from the AFM images.

The PCL scaffolds were analyzed by X-ray photoelectron spectroscopy (XPS, VG Multilab 2000, ThermoVG Scientific, Southent-on-Sea, UK) to obtain their elemental composition and chemical states introduced on the PCL surface by O₂ plasma etching. For each specimen, a compositional survey scan was acquired using a pass energy of 50 eV and core level spectra with a pass energy of 20 eV. Each specimen was
analyzed and averaged to obtain the reported atomic percent (at.%) values.

**Cell culture**

MC3T3-E1 (calvaria newborn mouse derived, ATCC CRL2593) cells were used to evaluate the cell response of the 3D PCL scaffolds by investigating cell proliferation, alkaline phosphatase (ALP) activity, and cell morphology through immunostaining and cell fixation. The cells were cultured in a humidified incubator at 37°C under 5% CO₂ atmosphere (Sanyo Electric, Osaka, Japan). The cells were cultured in minimum essential medium α modification (α-MEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% solution of 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, Basel, Switzerland). Medium was changed every 2 days, and the cells were detached with trypsin/EDTA (Sigma-Aldrich) and passaged at 90% confluence. The MC3T3-E1 cells used in this work were passaged three times.

**MC3T3-E1 cells seeding into 3D PCL scaffolds**

Before cell seeding, 3D PCL scaffolds were sterilized by immersing for 30 min in 70% ethanol and gently rinsed twice with Dulbecco’s phosphate buffered saline (DPBS, Welgene, Gyeongsan, Korea). Sterilized 3D PCL scaffolds were transferred into 48-well culture plates and cell suspensions were inoculated to the top center of the scaffolds, avoiding contact with the sides of the wells to improve cell seeding efficiency. After 3 h, the scaffolds were transferred to another 48-well culture plate and cell suspensions were inoculated to the top center of the scaffolds, avoiding contact with the sides of the wells to improve cell seeding efficiency. After 3 h, the scaffolds were transferred to another 48-well culture plate and each of the wells was filled with 500 µL fresh media.

**Cell proliferation**

The proliferation of MC3T3-E1 cells on 3D PCL scaffolds was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. In brief, 1×10⁵ cells/mL were seeded on scaffolds and the cells were incubated for 1, 3 and 5 days. At each of the sampling days, 10% MTT solution was added to the well and incubated at 37°C for 4 h for MTT formazan crystal formation. After developing the purple formazan colors by the reaction between metabolically active cells and tetrazolium salt, the medium and MTT solution were replaced with dimethyl sulfoxide (DMSO, JUNSEI, Tokyo, Japan) to dissolve purple formazan. The absorbance in each well was then measured using an EPOCH, absorbance microplate reader (BioTek Instruments, Winooski, VT, USA) at 540 nm.

**Cell differentiation**

The cells were cultured at a density of 1×10⁵ cells/mL and the medium was changed to α-MEM-containing 10 mM β-glycerophosphate (Sigma-Aldrich) and 50 µg/mL ascorbic acid (JUNSEI). After 7 and 14 days of culture in osteogenic induction medium, ALP was determined by quantifying the release of p-nitrophenol (p-NP) from p-nitrophenyl phosphate (p-NPP). The PCL scaffolds cultured with MC3T3-E1 cells were gently rinsed twice with DPBS (Welgene), lysed in 0.9% NaCl solution containing 0.2% Triton X-100 (Sigma-Aldrich) for 10 min and sonicated using a Vibra-Cell™ instrument (SONICS & Materials, Newton, CT, USA) for 1 min at 65 W on ice. The lysate was centrifuged at 2,500×g for 10 min at 4°C and the clear supernatant was incubated with p-nitrophenyl phosphate solution for 30 min at 37°C. The reaction was stopped by adding 600 µL of 1.2 N NaOH. The ALP activity was determined by measuring the absorbance at 405 nm using an EPOCH, absorbance microplate reader and normalized to the protein concentration. The protein concentration was determined by Bradford protein assay (Bio-Rad). The data are expressed as µmole p-NP/min/µg protein.

**Fixing of MC3T3-E1 cells for cell morphology observance**

The PCL scaffolds were prefixed with a mixed solution of 2.5% glutaraldehyde (Sigma-Aldrich) and 2.5% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) for 3 h, washed three times for 10 min each in the phosphate buffer, and then post-fixed in 1% osmium tetroxide (Sigma-Aldrich) for 30 min. The scaffolds were dehydrated in a graded series of aqueous ethanol solutions (70, 90, 95, and 100%) for 5 min each and then placed in hexamethyldisilazane solution (HMDS, Fluka, Buchs, Switzerland) to remove any alcohol. After 10 min, the solutions were removed and the scaffolds were allowed to air dry overnight at room temperature. To observe the SEM micrograph, the scaffolds were coated with gold thin layers using an automated ion sputter for 1 min.

**Live/dead cells assay**

Cell viability was assessed using a Molecular Probes Live-Dead cell staining kit (Biovision, Milpitas, CA, USA). MC3T3-E1 cells were seeded at a density of 3×10⁵ cells/mL on scaffolds in 48-well plates. After 2 days incubation, the culture media was removed from the wells and cells/scaffolds were rinsed three times with DPBS. Then staining solution (1 mM live-dye and 2.5 mg/mL of propidium iodide), 0.25 mL per well, was added, and the plates were cultured in an incubator for 20 min. Live cells (green) and dead cells (red) were visualized by fluorescence microscopy (NI-SS, Nikon, Tokyo, Japan) using a band-pass filter.

**Fluorescent labeling of actin cytoskeleton in MC3T3-E1 cell**

MC3T3-E1 cells cultured on scaffolds for one day were washed in DPBS and, fixed with freshly prepared 4% paraformaldehyde for 30 min. Fixed cells were washed with DPBS, followed by permeabilization with 0.1% Triton X-100 buffer. After 5 min of incubation with a blocking buffer containing 1% bovine serum albumin (1% BSA, 1×PBS), cells were incubated for 15 min with rhodamine-phalloidin (Molecular Probes, Life-Technologies, Eugene, OR, USA) diluted 1:100 in PBS. PCL scaffolds were washed three times with DPBS and, mounted on cover glass for microscopy with fluorescence mounting media 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). DAPI was visualized by fluorescence microscopy (NI-SS, Nikon, Tokyo, Japan) using a band-pass filter.
Fig. 1  *In vivo* scaffold implant surgery on the skull of a rabbit.

The skull of each rabbit was punched twice with a 10 mm trephine bur (a), and the scaffold plate was located on the punched side of the rabbit's skull (b).

Scaffold implant surgery on the skull of a domestic rabbit

Samples used in animal study were 3D PCL scaffolds with (height 1.8 mm and diameter 8 mm).

For the animal study, the following four groups were tested:

- **(A) Control non-treated PCL scaffolds with 150 µm pore size;**
- **(B) Non-treated PCL scaffolds with 350 µm pore size;**
- **(C) O₂ plasma-treated PCL scaffold (1 min) with 150 µm pore size;**
- **(D) O₂ plasma-treated PCL scaffold (24 min) with 150 µm pore size;**

This study was performed in accordance with the Institutional Guidelines for Experimental Animal Care and Use of Chonnam National University (CNU IACUC-H-2014-7). Ten domestic rabbits under general anesthesia were prepared using Xylazine (Rompun, Bayer Korea, Seoul, Korea) 10 mg/kg and Zoletil (Zoletil50, Virbac, Carros, France) 50 mg/kg. After fur removal to expose the skull sterilized with povidone-iodine (Potadine, Samil, Seoul, Korea), 2% lidocaine with 1:100,000 epinephrine was injected locally around the surgical site of the rabbits. Using a surgical blade the skulls of the rabbits were exposed, and the skull of each rabbit was punched twice with a 10 mm trephine bur. A scaffold plate was inserted into the punched side of each rabbit, and the surgical site was firmly sutured (Fig. 1). After irrigating the surgical site with normal saline solution, the recipient and donor sites were sutured with 3–0 Vicryl (Vicryl, Ethicon, Livingston, UK), and the skin was sutured with 3–0 Mersilk (Mersilk, Ethicon). After suturing, performing wound dressing with potadine, and confirming that the animals had awakened from anesthesia, they were shifted to the experimental animal laboratory. After surgery, a prophylactic antibiotic (Fortimicin, Young Jin Pharm, Seoul, Korea) and an anti-inflammatory drug (Fenaca, Hana Pharm, Seoul, Korea) were administered once a day for five days to prevent infection and to reduce the level of pain.

Micro-computed tomography (Micro-CT) analysis

At 2 weeks post-surgery, 10 rabbits were sacrificed by excess injection of pentothal sodium and the surgical sites were resected, including the scaffold plate from the rabbit’s skull with a fine bone cutter, and trimmed into a proper shape. The new bone formation was observed by a radiographic apparatus (Hi-Tex, Osaka, Japan) at 35 kV and 400 mA (2D). The X-ray source was set at 50 kV and 200 A with a pixel size of 17.09 mm. Exposure time was 1.2 s. Four hundred and fifty projections were acquired over an angular range of 180 degrees (angular step of 0.4 degrees). The image slices were reconstructed using 3D CT analyzer software (CTAN; Skyscan, Kontich, Belgium).

Histological evaluation of samples

The resected tissues were kept in formalin for two days and seeded in an EDTA solution. After paraffin embedding, 5 µm tissue sections were prepared and dyed with hematoxylin and eosin before observation *via* optical microscopy (Nikon, Melville, NY, USA). Using AperioImageScope v9.1 (ImageScope, Aperio Technologies, Vist, CA, USA) we obtained the histological digital images of the stained slice samples.

Histomorphometric analysis

The *in vitro* data were statistically analyzed using a student's *t*-test. *p*≤0.05 was considered significant (*). *p*≤0.01 was highly significant (**).

Histomorphometric analysis of the area of newly formed bone was carried out using Image Pro® Plus 6.0 (Media Cybernetics, Rockville, MD, USA). After tracing of the new bone, pixels of traced area were measured. The mean results were compared by one-way analysis of variance (ANOVA) using SPSS 20.0 TM program (IBM, Armonk, NY, USA). The differences were considered statistically significant at *p*<0.05.

RESULTS

Determination of optimized 3D PCL scaffold

Figures 2a–f show the SEM micrograph of 3D PCL scaffolds with different pore sizes and layout patterns. The fabricated 3D scaffolds demonstrate the 0°/45°, and 0°/90° strut layout pattern and 150, 250, and 350 µm pore size.

To determine the optimum scaffold architecture in the six scaffolds (Figs. 2a–f), proliferation of MC3T3-E1 cells was measured by an MTT assay after culture for 1, 3, and 5 days (Fig. 3). One day after cell seeding, a difference in cell proliferation was not observed. However, after 5 and 7 days, scaffolds with a 0°/45° strut layout pattern and 150 µm pore size demonstrated the highest cell proliferation among the all the groups. Based on this result, two scaffolds (45°/150 µm, 45°/350 µm) were selected as samples for further experiments such as plasma surface etching and *in vitro* and *in vivo* tests.
testing.

Surface characterization on 3D PCL scaffold after O2 plasma surface etching

In this experiment, we performed oxygen plasma etching on 3D PCL scaffolds selected in preliminary experiments to improve the surface roughness and surface chemical properties.

To observe the surface topology and roughness of the scaffolds before and after oxygen plasma etching, AFM analysis was performed. As shown in Fig. 4, the surface roughness was measured over an area 5×5 µm (Figs. 4a–c), and 10×10 µm (Figs. 4d–f). The untreated 3D PCL scaffold had a relatively smooth surface with
a roughness of 9.99 and 10.15 nm (Figs. 4a, d). As the plasma etching time increased, rough structures with peaks and valleys were formed with roughness 59.39, 61.65 nm (Figs. 4b, e), 233.17, and 246.07 nm (Figs. 4c, e), respectively. These results indicate that 3D PCL scaffolds were notably roughened by the oxygen plasma etching.

The XPS analysis was performed to identify the change in surface chemistry of 3D PCL scaffold surface after oxygen plasma treatment. The wide scan spectra and deconvoluted C1s peaks survey of XPS on the unetched and O2 plasma etched 3D PCL scaffolds for 1 and 24 min are shown in Figs. 5A, B. The contact angles of different PCL films are presented in Fig. 5C. The chemical compositions and relative peak areas of the various carbon and oxygen chemical bonds are shown.

Fig. 5  (A) XPS wide scan spectra of (a) pristine 3D PCL scaffold, (b) oxygen plasma etched 3D PCL scaffold for 1 min (c) oxygen plasma etched 3D PCL scaffold for 24 min and (B) high resolution C1s peaks of the top surface of the 3D PCL scaffolds before and after plasma etching in oxygen and (C). The water contact angles on the PCL films surface before and after oxygen plasma treatment.
in Table 1. These results demonstrated that the oxygen and carbon atomic ratio was increased from 0.36 to 0.45 when the plasma etching time increased from 1 min to 24 min. Furthermore, these oxygen-containing functional groups such as C-O and O=C-O have increased after O₂ plasma treatment whereas the carbon-carbon (C-C) or carbon-hydrogen (C-H) bond decreased. This can be attributed to cleavage of C-C and C-H bonds in the backbone of PCL.

As shown in Fig. 5C, the contact angle of the pristine PCL film was 54.8°±0.9°. After oxygen plasma etching for 1 and 24 min, contact angles were 22.3°±10.6° and 4.6°±1.6°, respectively. It is thought that the hydrophilicity increased with the increase in oxygen-related groups on the PCL film after the oxygen plasma etching. From the above results, O₂ plasma etching generated nano-size etched surface and oxygen-containing functional groups on the 3D PCL scaffolds surface.

**In vitro preosteoblast evaluation**

Figure 6 shows the cell proliferation and live/dead cell images on the unetched and etched PCL 3D scaffolds. Proliferation of MC3T3-E1 cells was determined by MTT assay after cultured for 1, 3, and 6 days on various scaffolds (Fig. 6A). In the scaffolds that underwent oxygen plasma etching for 1 min, a significant difference in cell proliferation was not observed. However, 45/150 scaffolds etched with oxygen plasma for 24 min showed the highest cell proliferation among the groups. These results suggest that cell proliferation is significantly affected by increasing surface roughness and hydrophilicity of the scaffold. All the group showed statistically significant differences between unetched and etched PCL scaffolds. Live/dead cell staining followed by evaluation with fluorescence microscopy showed the attached cells on the scaffold surface (Fig. 6B). More MC3T3-E1 cells were attached on the PCL scaffold etched with oxygen plasma than on the unetched PCL scaffolds. In the topmost layer of the scaffolds, fewer cells were observed on the unetched PCL scaffolds compared to the etched PCL scaffolds.

The MC3T3-E1 cell differentiation on the unetched and etched PCL scaffolds was evaluated by measuring ALP activity after 7 and 14 days. As shown in Fig. 7, differences in cell differentiation were not observed at 7 days. However, ALP activity of MC3T3-E1 cells grown on PCL scaffolds with etched oxygen plasma for 14 days was significantly increased compared to that on the unetched PCL scaffolds. It is possible that cell differentiation was significantly affected by oxygen-related groups, surface roughness, and hydrophilicity.

Figure 8A shows the cell morphology of MC3T3-E1 cells after culture for 30 min on the unetched and etched PCL scaffolds. The morphologies of the adhered cell on the oxygen plasma-etched PCL scaffolds showed
the appearance of filopodia spreading and lamellipodia extension and a larger circular shape compared with those on the unetched PCL scaffolds. Figure 8B shows fluorescent images after rhodamine-phalloidin and DAPI labeling of the actin cytoskeleton in MC3T3-E1 cells on unetched and etched PCL scaffolds for 2 days. DAPI stained the nucleus blue while the actin filaments were observed as red filamentous structures with rhodamine-phalloidin in the dual-dye staining of cells. After oxygen plasma etching, many cells were attached to the PCL fiber scaffold surface and the seeded cells exhibited superior larger cell morphology.

**Animal study for early new bone formation**

1. **Micro-CT evaluation**

   Figure 1 shows the 3D PCL scaffold implant surgery on the skull of a rabbit. The skull of each rabbits was punched twice with a 10 mm trephine bur, and the scaffold disk plate was implanted in the punched hole of the rabbit’s skull.

   Micro-CT analysis images demonstrated that new bone formation occurred throughout the 3D PCL scaffolds. New bone formation was rarely observed in non-treated 150 µm pore-sized scaffolds. On the other hand, some new bone was observed at the base of scaffolds where the skull was in contact (Fig. 9A). Similarly, in the case of non-treated 350 µm pore-sized scaffolds, new bone formation was observed at the base of scaffolds where the skull was in contact (Fig. 9B).
scaffolds, new bone formation was rarely observed except for some newly formed bone adjacent to the skull (Fig. 9B). However, in the 150 µm pore-sized scaffolds group etched with O₂ plasma for 1 min, new bone was formed along with the lattice of the 3D scaffold (Fig. 9C). For O₂ plasma etching time of 24 min, new bone formation showed similar results (Fig. 9D). These results revealed that O₂ plasma has a positive effect on new bone formation around the scaffold inner pores, as shown in Figs. 9C, D.

2. Histological analysis
Newly formed bone was rarely observed histologically in the non-treated 150 µm pore-sized scaffolds group, but inflammatory cells and fibro-vascular tissues were mainly observed with a small amount of new bone at the base of the scaffolds where they were in contact with the skull (Fig. 10A-a). In the non-treated 350 µm scaffolds group, fibro-vascular tissues were mainly observed, and there was no new bone formation (Fig. 10A-b). In contrast, for the 150 µm pore-sized scaffolds group etched with O₂ plasma for 1 min, new bone ingrowth into the inner pores of the 3D scaffold was observed. In addition, new bone formation up to 1 mm in height was observed from the base of the scaffolds (Fig. 10A-c). Similarly, new bone was formed along with the 3D lattice of 150 µm pore-sized scaffolds that underwent O₂ plasma treatment for 24 min (Fig. 10A-d).

3. Histomorphometric analysis
For histomorphometric analysis, we measured the area of newly formed bone using Image Pro® Plus 6.0 (Media Cybernetics) (Fig. 10B). In the non-treated 150 µm pore-sized scaffolds group, the area of newly formed bone was 735.7±6.3 pixels. In the non-treated 350 µm pore-sized scaffolds group, the area of newly formed bone was 248.3±48.3 pixels. In the 150 µm pore-sized 1 min O₂ plasma treatment scaffolds group, the area of newly formed bone was 3,322.4±89.9 pixels. In the 150 µm pore-sized 24 min O₂ plasma treatment scaffold group, the area of newly formed bone was 2,358.7±95.6 pixels. There were statistically significant differences between non-treated groups and O₂ plasma-treated groups (p<0.05); however, no significant differences were observed between non-treated groups and between O₂ plasma-treated groups (p>0.05).

DISCUSSIONS
3D PCL scaffolds fabricated by a RP technique have been used as bone substitute materials in bone tissue engineering applications[14]. Generally, their surface wettability and architecture play an important role in bone tissue regeneration and therefore, research has focused on optimizing these PCL scaffolds[37-40]. In the present study we investigated the effect of oxygen plasma etching and 3D scaffold design to optimize the 3D PCL scaffold performance, which was measured by preosteoblast cell proliferation, differentiation, and early new bone formation in vivo.

Microstructural properties of scaffolds such as pore size and interconnectivity regulate cellular responses such as adhesion, proliferation and differentiation[41]. It was reported that large pore size or porosity of the scaffold can allow for effective nutrient supply, gas diffusion and metabolic waste removal but lead to low cell attachment and intracellular signaling[42,43]. Consequently, appropriate pore sizes that support growth and maintenance of specific cells or tissue types have been extensively sought[44,45]. Many research groups have reported an optimum pore size of 100–400 µm for bone regeneration and 200–350 µm for osteoconduction[44]. Hence, we fabricated 3D PCL scaffolds with 0°/45°, and 0°/90 architecture and pore sizes of approximately 150,
250, and 350 µm, in this study (Figs. 2a–f). The 3D PCL scaffolds group with 45°/150 µm structure demonstrated the highest preosteoblast cell proliferation among all groups (Fig. 3).

Surface modification of 3D PCL scaffolds was performed using an oxygen plasma technique, to improve the surface wettability and roughness. After the oxygen plasma treatment for 1 and 24 min, the contact angle of the PCL scaffold surface was reduced from 54.8°±0.9° to 22.3°±10.6° and 4.6°±1.6°, respectively. Gupta et al. have shown that short exposure to oxygen plasma for 20 s could reduce the water contact angle of the PCL filament from 84° to 54° and also, the contact angle showed a decreasing trend with the increase in plasma exposure time40. In oxygen plasma, two processes occur simultaneously: etching of the polymer surface through the reactions of atomic oxygen with the surface carbon atoms, giving volatile reaction products; and the formation of oxygen-containing functional groups at the polymer surface through the reactions between the active species from the plasma and the surface atoms40. Therefore, it was possible to obtain the hydrophilic property for PCL films by oxygen plasma etching. This means that the high concentration of carbon-oxide sites on the surface of PCL films by oxygen plasma etching contributed to the adsorption hysteresis related to the high surface energy47.

The AFM observation showed that a random textured structure composed of pits, grooves, and protrusions was formed on all layer surfaces of 3D PCL scaffolds. As shown in Fig. 4A, the increment in surface roughness is due to the bombardment and oxidative chemical effect of the polymer surface induced by energetic ions and radicals present in the oxygen plasma etching48-50. Zhang et al. reported that oxygen plasma treatment introduced some polar groups to poly (p-phenylene benzobisoxazole) (PBO) fiber surfaces, enhanced surface roughness and changed surface morphologies of PBO fibers by plasma etching and oxidative reactions51.

The chemical structure of the PCL surface altered by oxygen plasma etching was identified by XPS. Oxygen plasma (1 and 24 min) induced oxidation of the PCL surface, where the O/C ratio increased from 0.32 to 0.36 and 0.45, respectively (Table 1). The oxygen functional groups formed by the oxygen plasma etching may be due to a post-oxidation of the radicals remaining on the PCL scaffold surfaces.

In order to investigate the effect of plasma surface modification and scaffold pore-sizes on cell-scaffold affinity, the cell proliferation and cell viability were tested using an MTT assay and live and dead cell staining (Figs. 6A, B). The cell proliferation results demonstrated that 24 min plasma-etched scaffolds showed the highest proliferation compared with the other scaffolds (Fig. 6A). In addition, as presented in Fig. 6B, higher numbers of live cells attached to the 24 min plasma-etched scaffold surface than the other samples. This might be explained by the dominant effect of increased surface roughness on osteoblast-scaffold affinity. However, ALP activity results demonstrated that there was no difference in cellular differentiation between 1 and 24 min plasma-etched scaffolds (Fig. 7). After oxygen plasma etching, an increase in cell proliferation and differentiation was observed on the rougher surfaces of the PCL scaffold compared to the less rough PCL scaffold surfaces. It is possible that oxygen plasma penetrated into PCL scaffolds and introduced hydrophilic functional groups onto surfaces of the inside pores of the scaffolds, and chemical modification directly improved hydrophilicity of PCL, and physical modification etched the surface of PCL scaffolds52.

Based on these observations, we conclude that oxygen plasma etching is capable of improving cell adhesion and proliferation on PCL scaffolds. We suggest that the hydrophilic surface of the PCL scaffold etched with oxygen plasma was more favorable for cell spreading and growth than the hydrophobic surface of unetched the PCL scaffold. The oxygen plasma etching thus improves the biocompatibility of biomaterial surfaces.

The ultimate goal in the present work was to fabricate the ideal scaffold for bone tissue replacement and the early new bone formation in the rabbit calvaria model. In the present work, oxygen plasma treatment was demonstrated to enhance the osteogenesis of MC3T3-E1 cells in vitro.

Furthermore, micro-CT analysis of each scaffold group, significant differences were observed between the non-treated scaffold groups and oxygen plasma-treated scaffold groups in the reconstruction of calvarial defects at 2 weeks after implantation (Figs. 9A–D). The inner pores of oxygen plasma-treated scaffolds were filled with new bone, as shown in Fig. 9C, D. This result indicated that oxygen plasma treatment can efficiently contribute to the early regenerating process of rabbit bone defects. When the plasma-treated scaffolds were compared with non-treated scaffolds in vivo, the effect of oxygen plasma on bone regeneration was clearly observed histologically. Oxygen plasma treatment increases the surface roughness of scaffolds, as shown in Fig. 4, and it may stimulate early bone regeneration. As presented in Figs. 10A (e–h), newly formed woven bone appeared in between internal pores of the scaffold and bone bonding between the implants and host bone was observed. Castillo-Dali et al. reported that when applied to rabbit skull defects, the roughened surface of polylactide acid (PLGA) membranes by the oxygen plasma improves bone regeneration at 2 months. In addition, incorporation of silicon dioxide layers onto PLGA membranes pre-treated with oxygen plasma promoted bone neoformation53.

Ardeshirylajimi et al. investigated the bone regeneration of human induced pluripotent stem cells (iPSC)-seeded plasma-treated polyether sulfone (PES) nanofibrous scaffolds after 8 weeks of implantation into critical-size calvarial defects in a rat model. Plasma-PES seeded with iPSCs induced the highest regeneration of bone defects among all groups54. An adequate roughness of the scaffold surface can enhance the cell affinity and the cells’ ability to bond to the cell-substrate interphase; and provide organized adhesion and differentiation of bone
cells to facilitate good tissue regeneration. In fact, surface topography has been well known to play a pivotal role in osteoblast adhesion, migration, and metabolism, thus modifying and controlling the osteoblastic differentiation. Based on our observations, we suggest that the oxygen plasma-treated scaffold surface provides a suitable microenvironment to improve bone regeneration.

CONCLUSIONS

In this work, the effects of oxygen plasma-etching and internal architecture (pore sizes, lay-down patterns) of the 3D PCL scaffolds on the preosteoblast cell proliferation and differentiation, and early new bone formation in rabbit calvaria were investigated. After the oxygen plasma-etching, surface roughness and hydrophilicity of PCL scaffolds were increased, and oxygen-containing groups were present on its surface. In vitro evaluations, we found that surface roughness and surface chemistry of 3D PCL scaffolds had a significantly positive effect on cell behaviors such as cell adhesion, proliferation, and osteogenic differentiation than that of the pore sizes. In addition, results of early new bone formation in the rabbit skull demonstrated that new bone ingrowth into the inner pores of the 3D scaffold on the oxygen plasma-etched groups was formed. Based on the results of in vitro and in vivo tests, oxygen plasma etching could be a useful technique for bone tissue engineering applications.

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