Biological Effect of Gas Plasma Treatment on CO₂ Gas Foaming/Salt Leaching Fabricated Porous Polycaprolactone Scaffolds in Bone Tissue Engineering

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1. Introduction

In bone tissue engineering, scaffold plays a key role in providing the appropriate matrices to regeneration of tissue and has to fulfill a few basic requirements such as high porosity, proper pore size, and surface properties permitting cell adhesion, differentiation, and proliferation [1]. Generally, the ideal scaffold should possess the properties of good biocompatibility, biodegradability with controllable degradation rate, easy fabrication, and sufficient mechanical properties [2]. Also, scaffolds must possess an open pore and a fully interconnected geometry in a highly porous structure with large surface area that will allow cell in-growth and an accurate cell distribution throughout the porous structure and will facilitate the neovascularization of the construct from the surrounding tissue [3].

Supercritical carbon dioxide (scCO₂) is widely used as a porogen to produce porous polymeric scaffolds. In addition, scCO₂ foaming technique is a very clean method for scaffolds production because it does not require the use of organic solvents to achieve porous scaffolds fabrication. A number of polymeric materials have been foamed by scCO₂ for tissue engineering purposes. For example, poly(D,L)lactide [4], poly(D,L)lactide-co-glycolide copolymers [4, 5], poly-ε-caprolactone [6–8], and polymethylmethacrylate [9]. Recently, a highly porous polymeric scaffolds with a well interconnected and homogeneous porous structure were prepared by the gas foaming/salt leaching method [10, 11]. The evolution of ammonia or carbon dioxide gases, as well as the leaching out of salt particulates from the solidifying polymer matrix, was found to produce macroporous scaffolds with pores ranging from 200 to 100 μm with no visible surface skin layer, which permits sufficient cell seeding within the scaffolds [10, 11].

Poly-ε-caprolactone (PCL) is an aliphatic biodegradable polymer with numerous potential applications in the tissue engineering application for bone and cartilage regeneration [12, 13]. The PCL is an excellent scaffold candidate due to its...
mechanical and structural properties and its ability to form a desired shape. The major limitation of PCL, however, is that it does not provide a desired environment for cell adhesion due to the lack of biological recognition sites and its intrinsic hydrophobicity [14].

Plasma surface modification techniques are used in biomedical engineering to modify the polymer surface to improve the adhesion, spreading, and proliferation of cells [15]. Also, surface modification with biomolecules is a typical strategy for improving the cellular response to conventional biomaterials. Biomaterial surfaces strongly affect the immune response, provide sites for cell adhesion, direct cell migration, and can trigger cell differentiation [16].

In this paper, we prepared porous PCL scaffolds via CO\(_2\) gas foaming and salt leaching process to apply to the bone tissue engineering. To improve the hydrophilicity and biocompatibility of porous PCL scaffolds, we performed oxygen or nitrogen plasma surface treatment.

2. Materials and Methods

2.1. Materials. PCL (\(M_w = 30 \text{ kDa} - 50 \text{ kDa}, T_m = 60^\circ\text{C}, \) and \(T_g = -60^\circ\text{C}\)) and NaCl were purchased from Sigma-Aldrich and Bio-Shop, respectively. The NaCl particles were ground and sieved to generate particles in the range of 150–212 𝜇m.

2.2. Fabrication of Porous PCL Scaffolds. The porous PCL scaffolds were prepared by CO\(_2\) gas foaming and salt leaching methods. PCL pellets were melted and mixed with PCL and NaCl in the ratios of 3:1 at 55°C. Subsequently, the samples were poured to Teflon mould with 2 cm diameter and 1 cm height. Gas foaming process was carried out by solubilizing CO\(_2\) within samples at 50°C and 8 MPa for 6 hrs. The pressure was quenched to the ambient very fast to allow for the formation of a bimodal pore structure. The release rate of scCO\(_2\) was 0.4 MPa/s. Figure 1 presents the schematic of CO\(_2\) gas foaming device. After CO\(_2\) gas foaming, the samples were immersed into distilled water (DW) for 1 day. DW was changed every 12 hrs.

2.3. Plasma Surface Treatment. The equipment for plasma surface modification is reported elsewhere [9]. The surface modification of PCL scaffold was carried out using a radio frequency (RF, 13.56 MHz) capacitively coupled plasma system (MINI PLASMA STATION, Korea). An oxygen and nitrogen plasma treatments were conducted to hydrophilic property and activate the PCL scaffolds surface. The oxygen plasma conditions were carried out at RF discharge power of 100 W, oxygen flow rate of 3 sccm, working pressure of 3.99 Pa, and treatment time of 60 s. For the nitrogen plasma, nitrogen flow rate was adjusted to 4 sccm.

2.4. Surface Characterization of PCL Scaffolds. The cross-sectional morphology of porous PCL scaffolds was observed by scanning electron microscopy (SEM; SEC, SNE-3200, Korea). After gas plasma treatment, the hydrophilicity of the PCL scaffolds surface was determined by contact angles using dynamic contact angle measurements (Contact-Angle, GS,

Surface Tech. Co. Ltd., Korea) and surface chemical compositions of the samples were analyzed in X-ray photoelectron spectroscopy (XPS, Multilab 2000 system, SSK, USA).

2.5. Cell Culture. MC3T3-E1(ATCC CRL-2953) cells, a clonal preosteoblast cell line derived from newborn mouse calvaria, were cultured in α-modified Eagle medium (Gibco), supplemented with 10% fetal bovine serum and 1% penicillin streptomycin and kept at 37°C in a saturated humid atmosphere containing 95% air and 5% CO\(_2\). Cells were detached with a trypsin/EDTA solution (Sigma-Aldrich) and suspended in α-modified Eagle medium (Gibco), supplemented with 10% fetal bovine serum and 1% penicillin streptomycin and kept at 37°C in a saturated humid atmosphere containing 95% air and 5% CO\(_2\). Cells were detached with a trypsin/EDTA solution (Sigma-Aldrich) and suspended in the correct medium. Before cell seeding, the samples were placed in 12 well culture plates and sterilized by soaking samples in 70% ethanol for 15 min. Then 1 × 10\(^5\) preosteoblast cells were seeded on sterilized samples.

2.6. Cell Proliferation. The proliferation of the cells was determined with MTT colorimetric assay. This test can detect the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) to formazan. The cell growth was stopped at 1, 3, and 6 days. After each time point, the cells were incubated in the medium supplemented with 10% bromide to allow the formation of water insoluble formazan crystals in 5% CO\(_2\) at 37°C for 4 hours. Then this product was dissolved in dimethyl sulfoxide (DMSO, Junsei) solution. 200 𝜇L aliquot of the solutions was

\(\text{CO}_2\) bomb → Pressur gauge → Vent

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<tr>
<th>Check valve</th>
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<td>Pressure gauge</td>
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Figure 1: The schematic of CO\(_2\) gas foaming device.
Figure 2: SEM cross-section images of (a) pristine, (b) O\textsubscript{2} plasma treated, and (c) N\textsubscript{2} plasma treated porous PCL scaffolds surface.

Figure 3: Contact angles of (a) pristine, (b) O\textsubscript{2} plasma treated, and (c) N\textsubscript{2} plasma treated PCL films surface.

Figure 4: XPS spectra of (a) pristine, (b) O\textsubscript{2} plasma treated, and (c) N\textsubscript{2} plasma treated porous PCL films surface.

aspirated and poured into a 96 well culture plate to measure optical densities (OD) with an ELISA reader (Thermal Fisher SCIENTIFIC), at a wavelength of 540 nm. Data (\(n = 3\)) were presented as means of OD values.

2.7. Cell Morphology Observation. After culturing for 24 h, cells were washed with phosphate-buffered saline (PBS) and then prefixed with a mixed solution containing 2.5% glutaraldehyde and 2.5% paraformaldehyde for 3 h, washed
three times for 10 min each in the phosphate buffer, and post-fixed in 1% osmium tetroxide for 30 min. The samples were dehydrated in a graded series of aqueous ethanol solutions (70%, 90%, 95%, and 100%) for 5 min each. The samples were then placed in hexa-methyl-di-silazane (Fluka) to remove any alcohol. After 10 min, the samples were removed and allowed to air dry overnight at room temperature. The sample was coated with a thin layer of gold using an automated sputter for 1 min. The MC3T3-E1 cells morphologies of each sample were observed by SEM (SNE-3200 M, SEC, Korea). The images were taken under an acceleration voltage of 30 kV.

2.8. Statistical Analysis. All the samples were cultured and assayed in triplicate at each time point specified. All the statistics are presented here as mean ± standard deviation. The results of the MTT assay were analyzed statistically using Student’s t-test. The statistical significance was considered at $P < 0.05$.

3. Results and Discussion

3.1. Surface Analysis of Porous PCL Scaffolds after Gas Plasma Treatment. Figure 2 shows the porous structure of various PCL scaffolds after gas foaming/salt leaching. After the gas plasma treatment, we observed the presence of open pore morphologies and high degrees of pore interconnectivity. The mean pore size of porous PCL scaffolds showed 427.89 μm. Indeed, the debonding between the soft (PCL) and hard (NaCl) domains may preferentially initiate the pore opening during gas bubble growth, allowing for the formation of pore interconnections, and hence the exposure of the microparticle-like porogen to the water [17].

As expected, the water drop remained on the top surface of the untreated PCL scaffold, while being absorbed into pores of plasma treated PCL scaffolds immediately. For the pristine PCL scaffolds, the value of contact angle showed $76.3 \pm 3.5^\circ$ ($n = 5$). However we could not measure the water contact angle on the gas plasma treated PCL scaffolds surface (data not shown). From these results, we could achieve a homogeneous functionalization of the interior surfaces of porous scaffolds and solved a problem of hydrophobic over topography, thus having a positive effect on the wettability improvement [19, 20].

To investigate the atomic element on PCL film surface after plasma treatment, XPS analysis is performed on different PCL films. Figure 4 shows the XPS survey spectra (low resolution) for PCL films with different exposure to nitrogen and oxygen plasma. In these spectra, carbon (C 1s at a BE of 285 eV), nitrogen (N 1s at a BE of 399 eV), and oxygen (O 1s at 533 a BE of eV) contributions can be clearly distinguished [21]. We can observe that new atomic elemental N is appeared after nitrogen plasma treatment. It was previously suggested that, under the O$_2$ plasma treatment, the scaffolds became more etched, and an increase in the concentration of polar components, for example, –C–O–, >C=O, and –COOH, on the surfaces resulted [22]. The different species present in a nitrogen plasma, such as N$_2$+, N$_3$ (excited), N, N$^+$, electrons, and UV radiation, interact with the surface of the polymer film and promote the formation of a large amount of free radicals, which play a relevant role in the functionalization process since they act as insertion points of active species [23].

3.2. Biological Evaluation for Gas Plasma Treated Porous PCL Scaffolds. Figure 5 shows the cell proliferation measured by a MTT assay after 1, 3, and 6 days of culture on O$_2$ plasma and N$_2$ plasma treated porous scaffolds in comparison with the pristine PCL porous scaffolds (control). As illustrated in Figure 5, viability of MC3T3-E1 cells on plasma treated PCL scaffolds increased during all culture times, compared to control group. Furthermore, it was observed that nitrogen plasma treatment is superior to oxygen plasma treatment. Gas plasma treatment offers an efficient method to chemically modify surfaces. These reactive species ionized by an electric discharge interact with material surfaces and lead to the incorporation of functional groups [24]. Gas plasma treatments applied to polymeric biomaterials modify not only their surface chemical composition but also roughness and wettability, which, as expected, can affect cell behavior as well [25–28].

Figure 6 shows the morphology of MC3T3-E1 cells cultured for 24 hours on pristine PCL scaffolds and plasma treated PCL scaffolds. Cell morphology on pristine and plasma treated PCL scaffolds was quite different. The MC3T3-E1 cells cultured on pristine PCL scaffolds appeared to be
small, spindle, irregular in shape, and separated from each other (Figure 6(a)). In contrast, MC3T3-E1 cells cultured on plasma treated PCL scaffolds presented higher density of adhered cells in close contact with each other, spread on the scaffolds surface (Figures 6(b) and 6(c)). In addition, the cells showed polygon and elongated morphology. It may be explained that cell adhesion and morphology were positively affected by O$_2$ and N$_2$ plasma treatment. As can be seen from the contact angle results (Figure 3), hydrophilic surface leads to good cell adhesion and morphology. Cells recognized not only topographical cues on the surfaces but also the surface chemistries, which can significantly influence their attachment and proliferation behavior [29]. Among the hydrophilic surfaces, differences in wettability significantly influence cell attachment but not spreading or cytoskeleton organization [29].

4. Conclusion

The porous PCL scaffolds with well-developed pores and interconnectivity were fabricated by CO$_2$ gas foaming and salt leaching process. The mean pore size showed 427.89 µm. It was found that the O$_2$ and N$_2$ plasma treatment provided O-containing and N-containing functional groups on the porous PCL scaffolds and consequently changed the PCL films surface extremely hydrophilic with contact angles of 5.40 and 8.6°, respectively. Cell viability results using MC3T3-E1 cells evaluated by MTT assay showed that the plasma treated PCL surfaces provide better cellular adhesion, enabling cell spreading and proliferation, indicating improved biological performance of the PCL scaffolds. This work may contribute to the improvement of the biological performance of polymeric biomaterials and increased feasibility of cell/polymer interaction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Tae-Yeong Bak and Min-Suk Kook contributed equally to this work.

References


