Hindawi Publishing Corporation
Journal of Nanomaterials
Volume 2014, Article ID 657542, 6 pages
http://dx.doi.org/10.1155/2014/657542

*Research Article*

# Biological Effect of Gas Plasma Treatment on $CO_2$ Gas Foaming/Salt Leaching Fabricated Porous Polycaprolactone Scaffolds in Bone Tissue Engineering

**Tae-Yeong Bak,[1] Min-Suk Kook,[2] Sang-Chul Jung,[3] and Byung-Hoon Kim[1]**

[1] *Department of Dental Materials, School of Dentistry, Chosun University, Gwangju 501-759, Republic of Korea*
[2] *Department of Oral and Maxillofacial Surgery, School of Dentistry, Chonnam National University, Gwangju 500-757, Republic of Korea*
[3] *Department of Environmental Engineering, Sunchon National University, Jeonnam, Suncheon 540-742, Republic of Korea*

Correspondence should be addressed to Byung-Hoon Kim; kim5055@chosun.ac.kr

Received 20 June 2014; Accepted 1 July 2014; Published 15 July 2014

Academic Editor: Seunghan Oh



Porous polycaprolactone (PCL) scaffolds were fabricated by using the $CO_2$ gas foaming/salt leaching process and then PCL scaffolds surface was treated by oxygen or nitrogen gas plasma in order to enhance the cell adhesion, spreading, and proliferation. The PCL and NaCl were mixed in the ratios of 3 : 1. The supercritical $CO_2$ gas foaming process was carried out by solubilizing $CO_2$ within samples at 50°C and 8 MPa for 6 hr and depressurization rate was 0.4 MPa/s. The oxygen or nitrogen plasma treated porous PCL scaffolds were prepared at discharge power 100 W and 10 mTorr for 60 s. The mean pore size of porous PCL scaffolds showed 427.89 $\mu$m. The gas plasma treated porous PCL scaffolds surface showed hydrophilic property and the enhanced adhesion and proliferation of MC3T3-E1 cells comparing to untreated porous PCL scaffolds. The PCL scaffolds produced from the gas foaming/salt leaching and plasma surface treatment are suitable for potential applications in bone tissue engineering.

## 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 ($scCO_2$) is widely used as a porogen to produce porous polymeric scaffolds. In addition, $scCO_2$ 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 $scCO_2$ 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 $\mu$m 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 kDa~50 kDa, $T_m$ = 60°C, and $T_g$ = −60°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 $\mu$m.

*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).

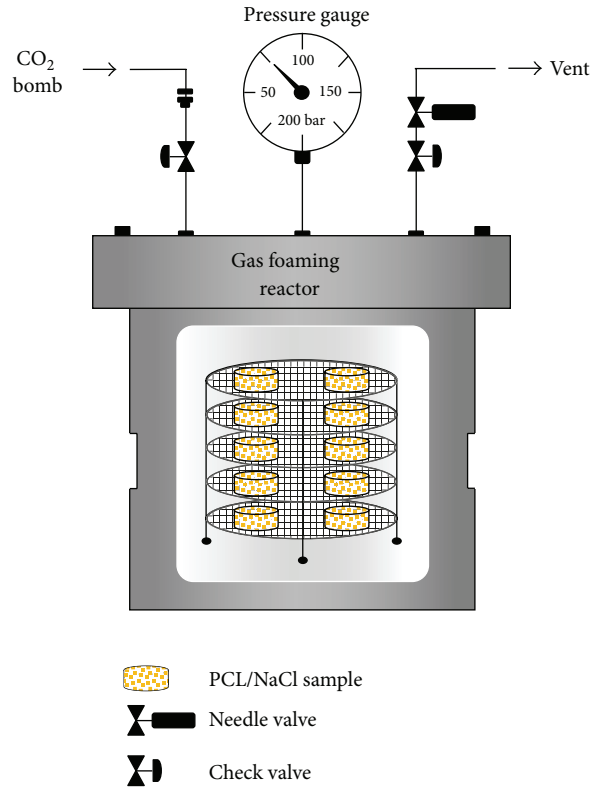



Figure 1: The schematic of $CO_2$ gas foaming device.

*2.5. Cell Culture.* MC3T3-E1(ATCC CRL-2953) cells, a clonal preosteoblast cell line derived from newborn mouse calvaria, were cultured in $\alpha$-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 $1 \times 10^5$ 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 $\mu$L aliquot of the solutions was

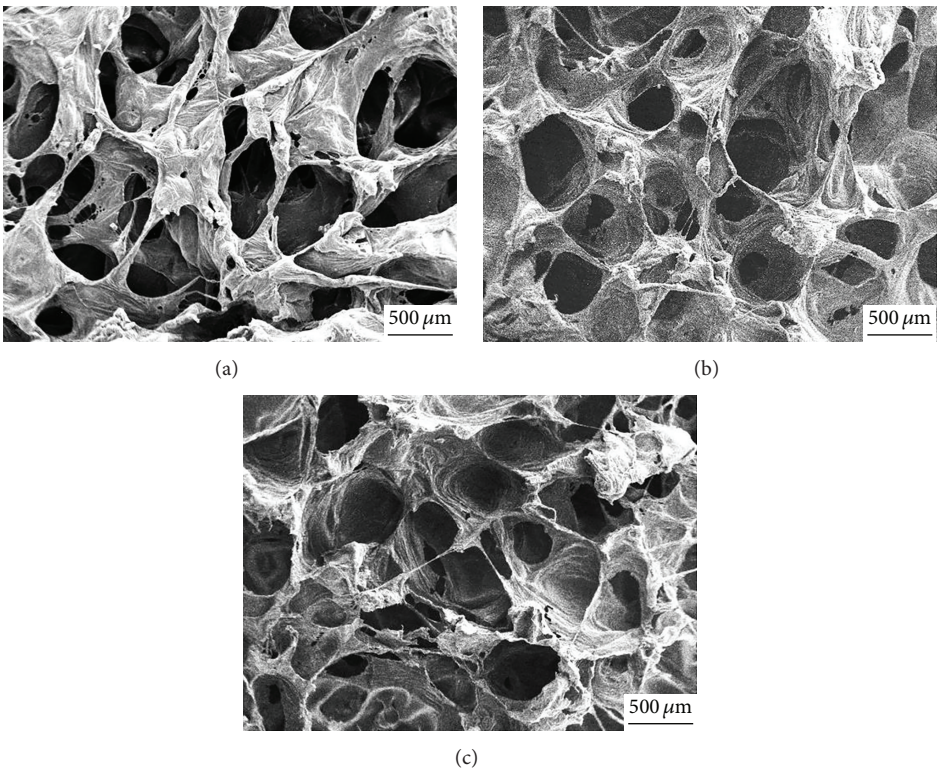



FIGURE 2: SEM cross-section images of (a) pristine, (b) $O_2$ plasma treated, and (c) $N_2$ plasma treated porous PCL scaffolds surface.

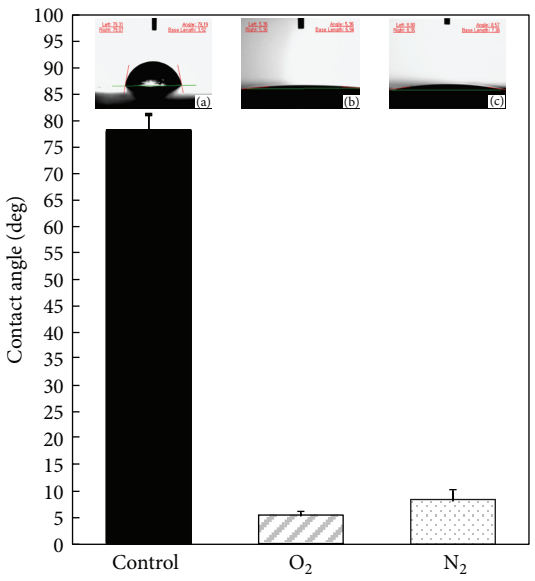



FIGURE 3: Contact angles of (a) pristine, (b) $O_2$ plasma treated, and (c) $N_2$ plasma treated PCL films surface.

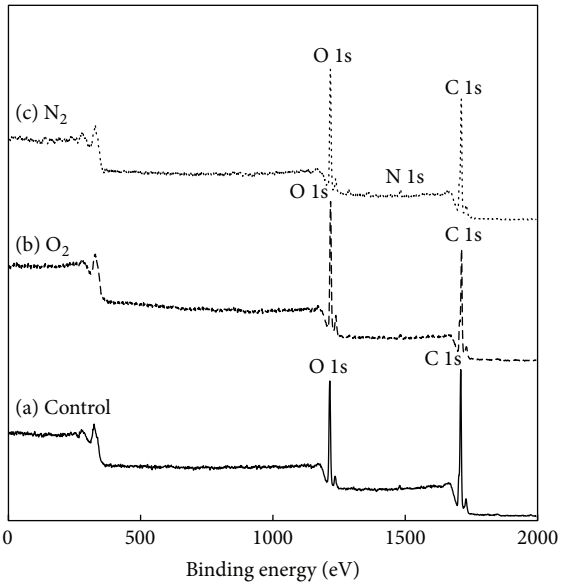



FIGURE 4: XPS spectra of (a) pristine, (b) $O_2$ plasma treated, and (c) $N_2$ plasma treated porous PCL films surface.

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 $\mu$m. 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 microparticulate 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 the PCL scaffolds surface. To investigate the effect on gas plasma treatment on the hydrophilicity of PCL scaffolds surface, we prepared the PCL films using a solvent casting, and then we have measured contact angles again. Figure 3 shows the different contact angles on the pristine, $O_2$ plasma treated, and $N_2$ plasma treated PCL films. For the pristine PCL film, the value of contact angle showed $79.2 \pm 4.5^{\circ}$ ($n = 5$). Untreated PCL scaffolds and PCL films surface show hydrophobic properties, whereas $O_2$ or $N_2$ plasma treated PCL film surfaces show the hydrophilicity. The action of the plasma promotes the formation of free radicals that can act as interlock points for active species (polar groups) [18]. Furthermore, depending on the gas and general conditions of the plasma treatment, it is possible to promote some surface etching/abrasion which can induce changes in surface topography, thus having a positive effect on the wettability improvement [19, 20].

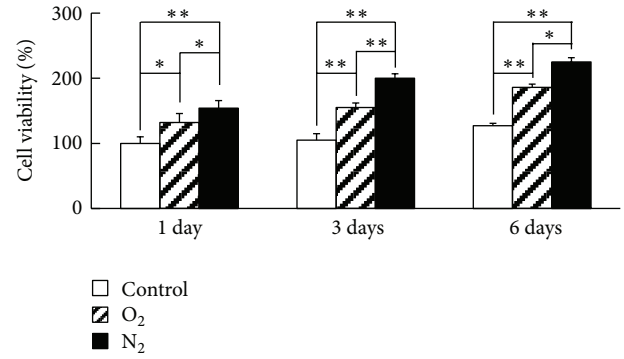
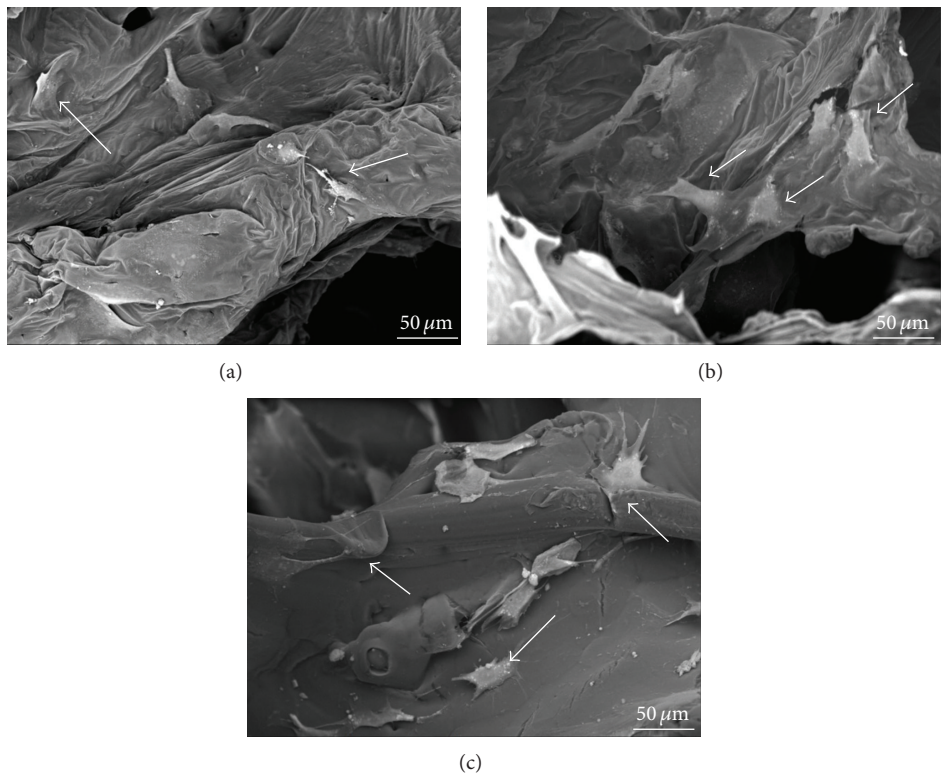



Figure 5: Cell proliferation of MC3T3-E1 cultured on pristine, $O_2$ plasma treated, and $N_2$ plasma treated porous PCL scaffolds surface for 1, 3, and 6 days ($^{*}P < 0.05$, $^{**}P < 0.01$).

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 1s 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 $N_2^+$, $N_2$ (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




(a) (b)

(c)

Figure 6: Cell morphology of the MC3T3-E1 cells grown on the PCL scaffolds for 24 h of culturing: (a) PCL, (b) PCL/$O_2$, and (c) PCL/$N_2$.

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 $\mu$m. 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

[1] H. Wang, Y. Li, Y. Zuo, J. Li, S. Ma, and L. Cheng, "Biocompatibility and osteogenesis of biomimetic nano-hydroxyapatite/polyamide composite scaffolds for bone tissue engineering," *Biomaterials*, vol. 28, no. 22, pp. 3338–3348, 2007.

[2] H. Zhang, C. Lin, and S. J. Hollister, "The interaction between bone marrow stromal cells and RGD-modified three-dimensional porous polycaprolactone scaffolds," *Biomaterials*, vol. 30, no. 25, pp. 4063–4069, 2009.

[3] A. J. Salgado, O. P. Coutinho, and R. L. Reis, "Bone tissue engineering: State of the art and future trends," *Macromolecular Bioscience*, vol. 4, no. 8, pp. 743–765, 2004.

[4] H. Tai, M. L. Mather, D. Howard et al., "Control of pore size and structure of tissue engineering scaffolds produced by supercritical fluid processing," *European Cells and Materials*, vol. 14, pp. 64–77, 2007.

[5] D. J. Mooney, D. F. Baldwin, N. P. Suh, J. P. Vacanti, and R. Langer, "Novel approach to fabricate porous sponges of poly(D,L-lactic-co-glycolic acid) without the use of organic solvents," *Biomaterials*, vol. 17, no. 14, pp. 1417–1422, 1996.

[6] M. J. Jenkins, K. L. Harrison, M. M. C. G. Silva, M. J. Whitaker, K. M. Shakesheff, and S. M. Howdle, "Characterisation of microcellular foams produced from semi-crystalline PCL using supercritical carbon dioxide," *European Polymer Journal*, vol. 42, no. 11, pp. 3145–3151, 2006.

[7] S. Cotugno, E. di Maio, G. Mensitieri et al., "Characterization of microcellular biodegradable polymeric foams produced from supercritical carbon dioxide solutions," *Industrial and Engineering Chemistry Research*, vol. 44, no. 6, pp. 1795–1803, 2005.

[8] I. Tsivintzelis, E. Pavlidou, and C. Panayiotou, "Biodegradable polymer foams prepared with supercritical $CO_2$-ethanol mixtures as blowing agents," *Journal of Supercritical Fluids*, vol. 42, no. 2, pp. 265–272, 2007.

[9] A. M. López-Periago, A. Vega, P. Subra et al., "Supercritical $CO_2$ processing of polymers for the production of materials with applications in tissue engineering and drug delivery," *Journal of Materials Science*, vol. 43, no. 6, pp. 1939–1947, 2008.

[10] L. D. Harris, B. S. Kim, and D. J. Mooney, "Open pore biodegradable matrices formed with gas foaming," *Journal of Biomedical Materials Research*, vol. 42, no. 3, pp. 396–402, 1998.

[11] Y. S. Nam, J. J. Yoon, and T. G. Park, "A novel fabrication method of macroporous biodegradable polymer scaffolds using gas foaming salt as a porogen additive," *Journal of Biomedical Materials Research A*, vol. 53, no. 1, pp. 1–7, 2000.

[12] E. D. Yildirim, H. Ayan, V. N. Vasilets, A. Fridman, S. Guceri, and W. Sun, "Effect of dielectric barrier discharge plasma on the attachment and proliferation of osteoblasts cultured over poly(ε-caprolactone) scaffolds," *Plasma Processes and Polymers*, vol. 5, no. 1, pp. 58–66, 2008.

[13] F. E. Wiria, K. F. Leong, C. K. Chua, and Y. Liu, "Poly-ε-caprolactone/hydroxyapatite for tissue engineering scaffold fabrication via selective laser sintering," *Acta Biomaterialia*, vol. 3, no. 1, pp. 1–12, 2007.

[14] S. L. Ishaug-Riley, L. E. Okun, G. Prado, M. A. Applegate, and A. Ratcliffe, "Human articular chondrocyte adhesion and proliferation on synthetic biodegradable polymer films," *Biomaterials*, vol. 20, no. 23-24, pp. 2245–2256, 1999.

[15] S. Jung, K. Lee, and B. Kim, "Biocompatibility of plasma polymerized sandblasted large grit and acid titanium surface," *Thin Solid Films*, vol. 521, pp. 150–154, 2012.

[16] J. Lee, F. Serna, and C. E. Schmidt, "Carboxy-endcapped conductive polypyrrole: biomimetic conducting polymer for cell scaffolds and electrodes," *Langmuir*, vol. 22, no. 24, pp. 9816–9819, 2006.

[17] S. H. Oh, I. K. Park, J. M. Kim, and J. H. Lee, "In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method," *Biomaterials*, vol. 28, no. 9, pp. 1664–1671, 2007.

[18] R. Molina, P. Erra, L. Julià, and E. Bertran, "Free radical formation in wool fibers treated by low temperature plasma," *Textile Research Journal*, vol. 73, no. 11, pp. 955–959, 2003.

[19] Y. M. Chung, M. J. Jung, J. G. Han, M. W. Lee, and Y. M. Kim, "Atmospheric RF plasma effects on the film adhesion property," *Thin Solid Films*, vol. 447-448, pp. 354–358, 2004.

[20] D. Hegemann, H. Brunner, and C. Oehr, "Plasma treatment of polymers for surface and adhesion improvement," *Nuclear Instruments and Methods in Physics Research B: Beam Interactions with Materials and Atoms*, vol. 208, pp. 281–286, 2003.

[21] M. R. Sanchis, O. Calvo, O. Fenollar, D. Garcia, and R. Balart, "Characterization of the surface changes and the aging effects of low-pressure nitrogen plasma treatment in a polyurethane film," *Polymer Testing*, vol. 27, no. 1, pp. 75–83, 2008.

[22] H. Lee, Y. Kang, S. Jeong et al., "Long-term aging characteristics of atmospheric-plasma-treated poly(ε-caprolactone) films and fibres," *Polymer Degradation and Stability*, vol. 96, no. 7, pp. 1204–1209, 2011.

[23] M. Dhayal, M. R. Alexander, and J. W. Bradley, "The surface chemistry resulting from low-pressure plasma treatment of polystyrene: the effect of residual vessel bound oxygen," *Applied Surface Science*, vol. 252, no. 22, pp. 7957–7963, 2006.

[24] A. M. C. Barradas, K. Lachmann, G. Hlawacek et al., "Surface modifications by gas plasma control osteogenic differentiation of MC3T3-E1 cells," *Acta Biomaterialia*, vol. 8, no. 8, pp. 2969–2977, 2012.

[25] K. S. Siow, L. Britcher, S. Kumar, and H. J. Griesser, "Plasma methods for the generation of chemically reactive surfaces for biomolecule immobilization and cell colonization—a review," *Plasma Processes and Polymers*, vol. 3, no. 6-7, pp. 392–418, 2006.

[26] H. Chim, J. L. Ong, J. Schantz, D. W. Hutmacher, and C. M. Agrawal, "Efficacy of glow discharge gas plasma treatment as a surface modification process for three-dimensional poly (D,L-lactide) scaffolds," *Journal of Biomedical Materials Research A*, vol. 65, no. 3, pp. 327–335, 2003.

[27] F. Mwale, H. T. Wang, V. Nelea, L. Luo, J. Antoniou, and M. R. Wertheimer, "The effect of glow discharge plasma surface modification of polymers on the osteogenic differentiation of committed human mesenchymal stem cells," *Biomaterials*, vol. 27, no. 10, pp. 2258–2264, 2006.

[28] T. B. F. Woodfield, S. Miot, I. Martin, C. A. Van Blitterswijk, and J. Riesle, "The regulation of expanded human nasal chondrocyte re-differentiation capacity by substrate composition and gas plasma surface modification," *Biomaterials*, vol. 27, no. 7, pp. 1043–1053, 2006.

[29] A. Martins, E. D. Pinho, S. Faria et al., "Surface modification of electrospun polycaprolactone nanofiber meshes by plasma treatment to enhance biological performance," *Small*, vol. 5, no. 10, pp. 1195–1206, 2009.