

Chondrocyte Infiltration and ECM Production on Surface-Treated PCL Scaffolds: Alkaline Hydrolysis versus Plasma Treatment

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Abstract—The objective of this study was to comparatively examine the responses of porcine chondrocytes to two different polycaprolactone (PCL) scaffolds whose surfaces were treated by alkaline hydrolysis and low pressure oxygen (O₂) plasma treatment, namely HPCL and plasma-treated PCL scaffolds, respectively. The surface morphology and the hydrophilicity of both scaffolds were evaluated by scanning electron microscopy (SEM) and a water contact angle measurement, respectively. The chondrocytes cultured on each scaffold were assessed for their proliferation, cartilage-specific gene expression, cell infiltration, and extracellular matrix (ECM) synthesis after a 21-day culture period. The scanning electron micrographs revealed the increased roughness of both HPCL and plasma-treated PCL scaffolds compared with the untreated PCL scaffold. The measured water contact angle of the plasma-treated PCL scaffold appeared much smaller than that on the HPCL scaffold. The chondrocytes cultured on the HPCL and plasma-treated PCL scaffolds exhibited an insignificant difference in cell proliferation. The expression of type II collagen and aggrecan mRNA found on both surface-treated scaffolds was not much different, either. Nevertheless, the histological results demonstrated that the chondrocytes on the plasma-treated PCL scaffold could more thoroughly infiltrate into the inner parts of the scaffold than those on the HPCL scaffold. Furthermore, a greater ECM production was observed on the plasma-treated PCL scaffold.

Index Terms—Alkaline hydrolysis, chondrocytes, extracellular matrix, plasma treatment, polycaprolactone.

I. INTRODUCTION

A number of aliphatic polyesters, e.g., poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and polycaprolactone (PCL), have been studied as scaffolds for cartilage tissue engineering applications due to their biodegradability, biocompatibility and non-cytotoxicity [1], [2]. Hydrophobicity of these polymers, however, may hinder cell attachment and growth on their surfaces. Surface modifications of polymeric scaffolds have been attempted to enhance the hydrophilicity of the scaffolds in order to promote cell adhesion, proliferation and functions. As reported in the literature, the NaOH-treated poly (lactic-co-glycolic acid) (PLGA) scaffolds were found to enhance chondrocyte functions more effectively than the untreated PLGA scaffolds [3]. Furthermore, the plasma treatment of poly (L-lactide) was found to enable the penetration of

mouse 3T3 fibroblasts into the poly (L-lactide) scaffolds and facilitate cell proliferation [4]. Plasma treatment has been widely used since it rarely involves chemical reagents harmful to cells and biological tissues. Moreover, it induces both physical and chemical changes in the beneficial bulk properties of the materials, e.g., surface chemical composition, roughness and wettability, which directly govern the cellular behaviors and functions [5], [6].

In this study, a comparative evaluation was performed on two differently surface-treated polycaprolactone (PCL) scaffolds: by alkaline hydrolysis using NaOH and by low pressure oxygen (O₂) plasma treatment. The responses of porcine chondrocytes to the alkaline hydrolyzed-PCL (HPCL) and low pressure oxygen (O₂) plasma treated PCL (plasma-treated PCL) scaffolds were assessed for their proliferation and cartilage-specific gene expression using Alamar blue assay and RT-PCR analysis. The cell infiltration and extracellular matrix (ECM) production were also investigated by histological analysis.

II. MATERIALS AND METHODS

A. Preparation of Porous PCL and HPCL Scaffolds

Polycaprolactone (PCL) ($\bar{M}_w = 80,000$ g/mol) was supplied from Sigma-Aldrich Corporation in a pellet form. Typically, PCL pellets were initially dried overnight at room temperature under vacuum prior to the scaffold fabrication using a supercritical carbon dioxide technique. 5 g of the dried sample was loaded into a cylindrical vessel which was heated at 60°C for 10 min. Subsequently, the vessel was filled with CO₂ at 15 MPa, and the polymer was soaked for 3 h. At the end of the process, the whole system was rapidly depressurized at 1.2 cc/sec, yielding the porous PCL scaffold with an average pore size in the range of 150-250 μ m. Alkaline hydrolyzed-PCL (HPCL) scaffold was prepared by the hydrolysis of the PCL scaffold with 1N NaOH solution at 45°C for 6 h. The alkaline-treated scaffold was washed thoroughly in de-ionized water and then freeze-dried overnight.

B. Preparation of Plasma-Treated PCL Scaffold

Plasma treatment was carried out on a low pressure RF discharge (model PDC-002, Harrick) which is sustained in pure O₂. The PCL scaffold was placed on the glass plate positioned approximately 7.5 cm below the plasma reactor in a direction orthogonal to the plasma source head. The chamber was evacuated below a pressure of 205 mTorr and then filled with pure oxygen. The PCL scaffold was treated

Manuscript received January 4, 2013; revised March 14, 2013. This work was supported by National Metal and Materials Technology Center.

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at 30W for 30 min, yielding plasma-treated PCL scaffold.

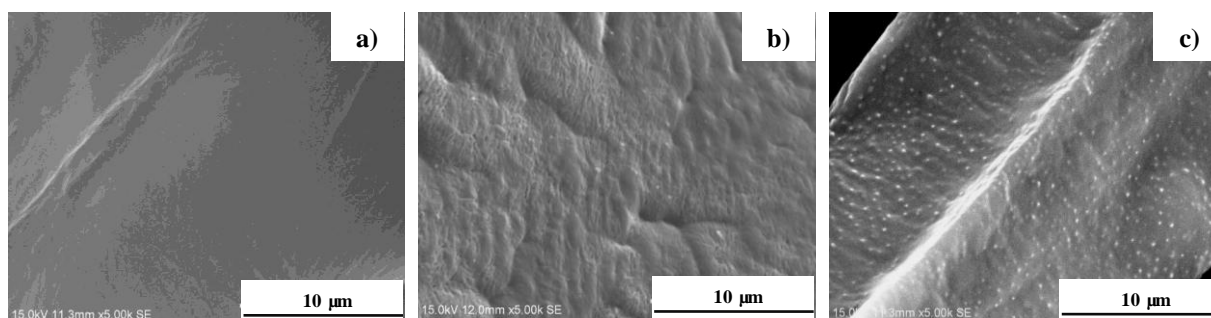


Fig. 1. SEM images of internal pore surfaces of various scaffolds: a) PCL, b) HPCL, c) plasma-treated PCL, original magnification x5000.

C. Surface Morphology Examination

A scanning electron microscope (SEM, Hitachi S-3400N) was used for the observation of the internal pore morphology of the untreated and surface-treated PCL scaffolds.

D. Water Contact Angle Measurement

The hydrophilicity of all the PCL scaffolds was comparatively evaluated by means of water contact angle measurement using a sessile drop technique with an optical bench-type contact angle goniometer (model 100-00-220, Rame-Hart, USA).

E. Cell Culture

The sterilized 8 mm sample discs were placed into 24-well culture plates. Each scaffold was seeded with porcine chondrocytes at concentration of 1.5×10^6 cells/specimen and then incubated overnight to allow the cells to attach. Afterwards, the cell-seeded scaffolds were cultured in 24-well culture plates incubated under 5% CO₂ atmosphere at 37°C for 21 days. The culture medium was regularly replaced every 2 days.

F. Cell Proliferation Assay

Cell proliferation on each scaffold was assessed by Alamar blue assay, which is based on the detection of metabolic activity of the cultured cells. After a 21-day incubation period, the chondrocytes cultured on each scaffold were further incubated in a medium containing resazurin dye for 4 h. Aliquots of 200 µl of each medium were subsequently read at the fluorescence wavelength of 530/590 nm.

G. RNA Extraction and RT-PCR Analysis

In brief, after a 21-day culture period, total RNA was extracted from the chondrocytes cultured on each scaffold using TRIZOL reagent (Invitrogen) by following the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 2 µg of RNA using Prime RT Master synthesis Kit (GeNet Bio) in 20 µl reaction. PCR analysis was performed to determine the expression of cartilage-specific genes, i.e., type II collagen and aggrecan. The mRNA levels of 18S rRNA were used as internal controls. The PCR products were identified by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The band intensity of type II collagen and aggrecan was assessed using Image J program. The data were normalized by the 18S rRNA levels in each

sample.

H. Histological Analysis

After culture, the cells were fixed with 2% paraformaldehyde solution, embedded in paraffin, and sectioned (7 µm thickness). The sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome to observe cell infiltration and total collagen, respectively.

III. RESULTS AND DISCUSSION

A. Surface Morphology

The SEM images of internal pore surfaces of each scaffold are shown in Fig. 1. The surface of the untreated PCL scaffold was fairly smooth (Fig. 1 a). In contrast, the alkaline hydrolyzed and low pressure O₂ plasma treated PCL scaffolds revealed rough pore surfaces (Fig. 1 b) and 1 c). Specifically, the plasma-treated PCL scaffold developed nodule-like structure, which was thoroughly distributed on its pore surface. This pore surface topography was caused by plasma etching.

B. Water Contact Angle

To evaluate the hydrophilicity of the scaffolds, the water contact angles on the surface of each scaffold were measured by a sessile drop technique; the results are demonstrated in Table I. A slight decrease in water contact angle was noted after the PCL scaffold was alkaline hydrolyzed by NaOH. The plasma surface treatment, however, considerably increased the surface hydrophilicity of the PCL scaffold; the water contact angle was reduced from $117.60 \pm 3.40^\circ$ on the untreated PCL scaffold to $48.03 \pm 12.89^\circ$ on the plasma-treated PCL scaffold. It was suggested that, under the O₂ plasma treatment, the scaffold surface became etched, and an increment in the concentration of polar components, e.g., -C-O-, >C=O, and COOH, on the surface was yielded, leading to the enhancement of surface hydrophilicity [6].

TABLE I: WATER CONTACT ANGLES MEASURED ON VARIOUS PCL SCAFFOLDS

Sample code	Water contact angle (X°)*
PCL	117.60±3.40
HPCL	110.50±6.54
plasma-treated PCL	48.03±12.89

* tested onto three different sites of each sample.

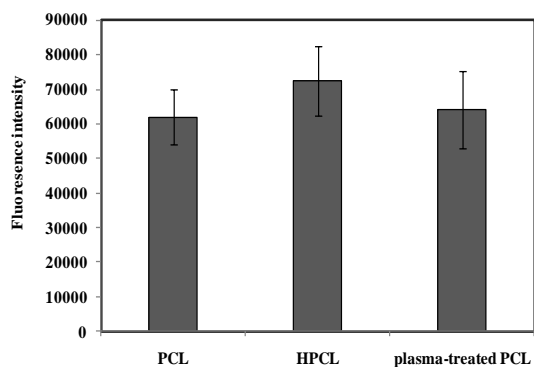


Fig. 2. Cell proliferation on various PCL scaffolds at a 21-day culture period.

C. Cell Proliferation

To investigate the cell growth on each scaffold, a cell proliferation assay was carried out using Alamar blue assay. As revealed in Fig. 2, the proliferation of chondrocytes cultured on the untreated PCL and surface-treated PCL scaffolds, i.e., HPCL and plasma-treated PCL, showed insignificant differences after being cultured for 21 days.

D. Cartilage-Specific Gene Expression

The cartilage-specific gene expression in the chondrocytes cultured on each scaffold was determined by RT-PCR analysis. As demonstrated in Fig. 3, compared to those cultured on the untreated PCL, the chondrocytes cultured on the HPCL and plasma-treated PCL scaffolds considerably enhanced their mRNA expression of cartilage-specific genes, i.e., type II collagen and aggrecan. The surface roughness and altered surface chemical compositions (data not shown) resulted from both surface treatments stimulated the chondrocytes to maintain their cellular phenotype and functions more properly.

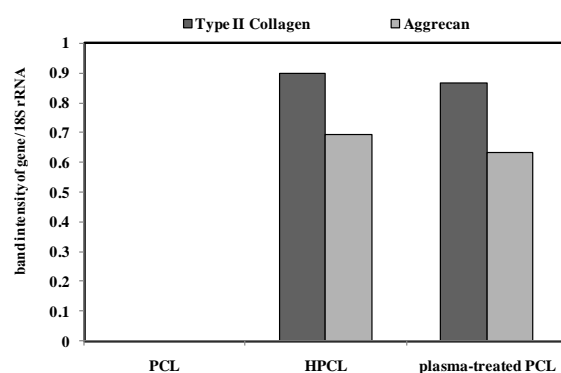


Fig. 3. Band intensity of mRNA expression of type II collagen and aggrecan secreted from chondrocytes cultured on various PCL scaffolds at a 21-day culture period.

However, there were no significant differences in the type II collagen and aggrecan band intensities determined between the HPCL and plasma-treated PCL scaffolds.

E. Cell Infiltration and Extracellular Matrix Production

Fig. 4 shows the histological results of the sectioned specimens at a 21-day culture period. Apparently, the porcine chondrocytes proliferated and covered on the top surfaces of the HPCL and plasma-treated scaffolds more densely than those found on the untreated PCL scaffold. In addition, the extracellular matrix (ECM) was obviously detected on the top surfaces of those treated scaffolds. This suggested that the increased roughness of both treated scaffolds could promote the cell adhesion, growth and functions, resulting in increased cell proliferation and ECM secretion.

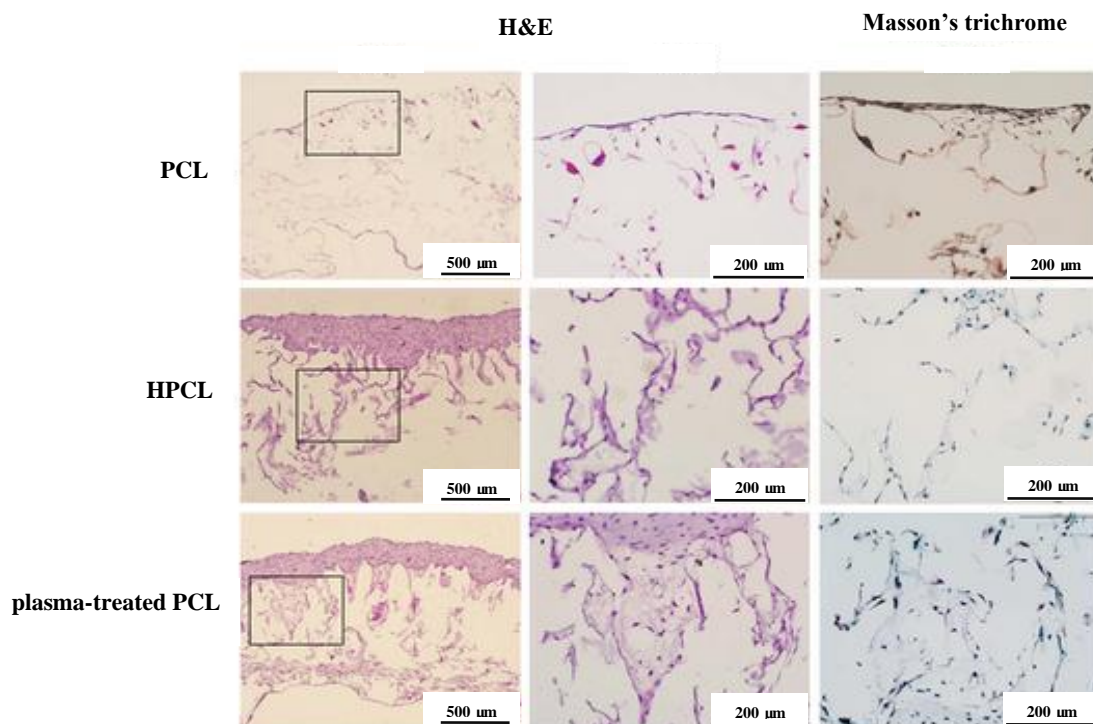


Fig. 4. Histological appearance of cross-sections of various PCL scaffolds at a 21-day culture period. The sections were stained with H&E or Masson's trichrome (blue color indicates total collagen deposition).

The chondrocytes also infiltrated more deeply into the surface-treated scaffolds than those cultured on the untreated PCL scaffold. The Masson's trichrome staining results, however, displayed the densest distribution of total collagen deposition in the plasma-treated scaffold. These results were in good accordance with the results on the water contact angle measurement in which the plasma-treated PCL scaffold appeared most hydrophilic. The enhanced surface hydrophilicity of the scaffold could facilitate the cell infiltration through the inner scaffold more profoundly, and consequently more ECM content was produced and distributed throughout the scaffold. The surface topography and hydrophilicity have been shown to influence the interaction between the porcine chondrocytes and the PCL scaffolds.

IV. CONCLUSION

Two different surface-treated PCL scaffolds, alkaline hydrolyzed and low pressure O₂ plasma treated PCL scaffolds, were found to promote the type II collagen and aggrecan gene expression of the porcine chondrocytes. However, compared with those cultured on the NaOH-treated PCL scaffold, the chondrocytes cultured on the plasma-treated PCL scaffold infiltrated through the scaffold more profoundly and produced more ECM. These results demonstrated that the superior hydrophilicity of the plasma-treated PCL scaffold seemed to provide the most favorable environment for the chondrocytes to function and maintain their differentiated phenotype.

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