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Contributed Paper

In Vitro Cultivation of Porcine Chondrocytes on Fully Surface-modified Polycaprolactone Scaffold: Static versus Dynamic Cultures

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ABSTRACT

Tissue engineering has been anticipated to be a promising therapeutic approach for cartilage damage. Hence, the development of a suitable scaffold for reconstruction of neocartilage is one of the key issues. In this study, polycaprolactone (PCL) was used as a scaffold in two different cell culture processes: static and dynamic cultures. The former was simply conducted in a cell culture plate, while the latter was performed in an in-house-built flow perfusion bioreactor at a flow rate of 0.1 ml/min. In the preparation of the fully surface-modified PCL scaffold, PCL pellets were first alkaline hydrolyzed prior to fabrication by a high pressure supercritical CO_2 technique. This resulted in the hydrolyzed PCL (HPCL) scaffold with pore sizes ranging from 150 to 250 μm, whose surface was further subjected to an oxygen plasma treatment. The surface morphology, wettability, and chemical composition of the resulting scaffold were analyzed by scanning electron microscopy (SEM), water contact angle measurement, and X-ray photoelectron spectroscopy (XPS), respectively. It was found that the surface roughness and hydrophilicity of the PCL scaffold were drastically enhanced after the treatments. The proliferation of porcine chondrocytes cultured on the fully surface-modified PCL scaffold was found not to be different under both static and dynamic culture environments. However, the dynamic culture could promote the cells cultivated on the scaffold to function more effectively with a higher production and accumulation of extracellular matrix and a greater secretion of two important cartilage specific components, type II collagen and aggrecan. The total GAG content produced by the cells cultured in the bioreactor was about 95 μg/scaffold, while that produced in the well plate was only about 28 μg/scaffold. In addition, it was noted that the fully surface-modified PCL scaffold facilitated the infiltration of the cells down through the scaffold. The use of the suitably surface-modified PCL scaffold, together with the application of a flow perfusion bioreactor, potentiated the *in vitro* generation of cartilaginous extracellular matrix components.

Keywords: polycaprolactone, chondrocytes, alkaline hydrolysis, plasma treatment, flow perfusion bioreactor, static culture

1. INTRODUCTION

The most common treatment for patients with severe cartilage damage is a surgical transplantation, due to the low proliferation of cartilage cells and its avascular structure which result in a low self-healing potential after injury [1]. Numerous technologies have been employed to heal damaged articular cartilage, but none of them achieves a full restoration of cartilage function or returns the damaged tissue to its normal state. Recently, cartilage tissue engineering has been extensively studied and demonstrated a great promise for repairing of articular cartilage damage or defects by the generation of neocartilage [2, 3]. To maintain normal cartilage structure and function, chondrocytes, the only resident cell type in cartilage, basically secrete a high amount of extracellular matrix (ECM) and cartilage-specific components, such as type II collagen and aggrecan [4]. Thus, the biological properties of chondrocyte-seeded constructs in cartilage tissue engineering must be similar to those of the native tissue [5-7]. For instance, they should allow good cell spacing and maintain chondrocytic phenotypes of the seeded cells with the subsequent formation of ECM which is essential for cartilage reconstruction [7].

A number of different three-dimensional (3D) porous scaffolds have been widely used *in vitro* to support the adhesion, growth, and functions of chondrocytes. They were either polymeric or composite materials [8-11]. In our previous work, the fully surface-modified polycaprolactone (PCL) scaffold was prepared by initially alkaline-hydrolyzing PCL pellets prior to scaffold fabrication by a high pressure supercritical CO_2 technique and then treating the surface of the hydrolyzed PCL scaffold with an oxygen plasma treatment [12]. The fully surface-modified PCL scaffold has demonstrated its great potential to satisfactorily induce cartilaginous tissue formation as assessed by chondrocyte proliferation assay, formation of GAG content, and expression of chondrogenic phenotype markers. However, these results were acquired from the static culture system which had certain shortcomings, such improper nutrient transfer, insufficient oxygen concentration, and metabolic waste accumulation [13, 14].

A bioreactor is a normal device used in a dynamic culture system that allows a convective transport of oxygen and nutrients to cells seeded on a scaffold [15-17]. This overcomes the drawbacks typically found in the static culture condition. The bioreactor not only provides the fluid flow but also compression load and shear stress mimicking *in vivo* cellular environments [18-22]. The mechanical stimulation in the bioreactor culture system was found to activate chondrocyte growth and the synthesis and accumulation of ECM which were the key procedures in cartilaginous tissue reconstruction [23].

In the present study, the ability to induce cartilaginous tissue formation of cartilage cells cultured on the fully surface-modified PCL scaffold was further investigated in a comparative study between the static and dynamic cell culture processes. The dynamic culture was conducted in an in-house-built flow perfusion bioreactor at a flow rate of 0.1 ml/min, while the static culture was carried out in polystyrene cell culture well plates. The porcine chondrocyte proliferation, cartilage-specific gene expression, and accumulation of ECM components on the scaffold were comparatively evaluated in both culture systems.

2. MATERIALS AND METHODS

2.1 Fabrication of Fully Surface-modified PCL Scaffold

Firstly, PCL ($\overline{Mn}$ = 80,000 g/mol, Sigma-Aldrich, Singapore) pellets were hydrolyzed by 6 M NaOH at 50 °C for 5 h and then vacuum dried overnight at room temperature to obtain hydrolyzed PCL pellets which were designated as HPCL pellets. Supercritical CO_2 ($scCO_2$) technique was used in the fabrication of 3D porous HPCL scaffold. In brief, 5 g of dried HPCL pellets was loaded into a cylindrical vessel and heated at 60 °C for 10 min before being foamed with CO_2 at 15 MPa for 3 h. The CO_2 was finally depressurized at 1.2 cc/s, to yield the porous HPCL scaffold. The surface of the resulting scaffold was further plasma-treated to enhance the surface hydrophilicity of the HPCL scaffold by placing HPCL scaffold specimens in a low-pressure oxygen plasma chamber (model PDC-002, Harrick Scientific Corp., Ossining, NY, USA), which was evacuated below a pressure of 205 mTorr. After being treated at 30 W for 30 min, the scaffold was designated as plasma-treated HPCL scaffold.

2.2 Scanning Electron Microscopy

The pore structure and internal pore morphology of the plasma-treated HPCL scaffold were examined by scanning electron microscopy (SEM, Hitachi S-3400N, Japan) at an accelerating voltage of 15 kV. The specimen was first cut by a surgical blade after being immersed into liquid nitrogen and later coated with gold for 120 s using a sputter-coater before SEM analysis.

2.3 Water Contact Angle Measurement

To evaluate the hydrophilicity of the scaffold, the water contact angle measurement was performed using a sessile drop technique at room temperature. Typically, a droplet of approximately 10 µl deionized water was deposited onto a given surface site of a scaffold specimen, and the water contact angle was then measured directly with an optical bench-type contact angle goniometer (model 100-00-220, Rame-Hart, Mountain Lakes, NJ). Three different sites per specimen were randomly tested, and the scaffold was tested in triplicate (n=6, totally). The water contact angles measured were finally averaged and reported as mean ± standard deviation (mean±SD).

2.4 X-ray Photoelectron Spectrometry

The surface chemical composition of the plasma-treated HPCL scaffold was investigated by an X-ray photoelectron spectrometer (XPS; AXIS ULTRA^{DLD}, Kratos analytical, Manchester, UK) using a hemispherical electrostatic energy analyzer and an Al K_α (1.4 keV) X-ray source. The base pressure in the XPS analysis chamber was about 5.0×10^{-9} Torr. The photoelectrons were detected with the hemispherical analyzer positioned at an angle of 45° with respect to the normal to the specimen surface.

2.5 Preparation of Porcine Chondrocytes

Porcine articular cartilage sections were isolated from the knee joints of a newborn pig. The collected knee cartilage was minced into pieces and digested in 0.2% (w/v) collagenase (Sigma-Aldrich, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM; Gibco/Life Technologies, Grand Island, NY) at 37 °C for 6 h. The chondrocyte pellet was collected after the mixture was centrifuged at 1000 rpm for 5 min and washed twice with PBS. The cell pellet was re-suspended in DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco) together with penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco) and plated in 100 mm TC-Treated Culture Dish

(Corning, NY). The porcine chondrocytes were subsequently maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO_2.

2.6 Cell Culture

Scaffold specimens with a diameter of 8 mm and thickness of 2 mm were sterilized by gamma radiation and then placed in 24-well culture plates before being seeded with porcine chondrocytes (1.0×10^6 cells/scaffold). The cell-seeded specimens were incubated at 37 °C for 3 h in a 5% CO_2 incubator with a humidified atmosphere to allow the cells to attach on the scaffold specimens and then transferred into new culture plates to be further cultured for 5 more days. In the static cell culture process, after 5-day culture period, the cell-seeded scaffolds were still continuously incubated in the 24-well plates for 21 more days with a replacement of the culture medium every other day. In the dynamic cell culture process, each cell-seeded scaffold was transferred into a bioreactor chamber in the perfusion culture system which consisted of nine bioreactor chambers. Each chamber was directly connected with each medium reservoir (Duran, Germany) through a Masterflex® C-FLEX® tube (Cole-Parmer, USA) and with Masterflex® peristaltic pump (Cole-Parmer, USA), as shown in Figure 1. A constant flow rate of 0.1 ml/min was applied to control the medium flux flowing through the cell-seeded scaffolds in the flow perfusion bioreactor system which was maintained at 37 °C under humidified atmosphere of 5% CO_2 for 16 days. Each reservoir contained 30 ml of culture medium which was changed every 4 days.

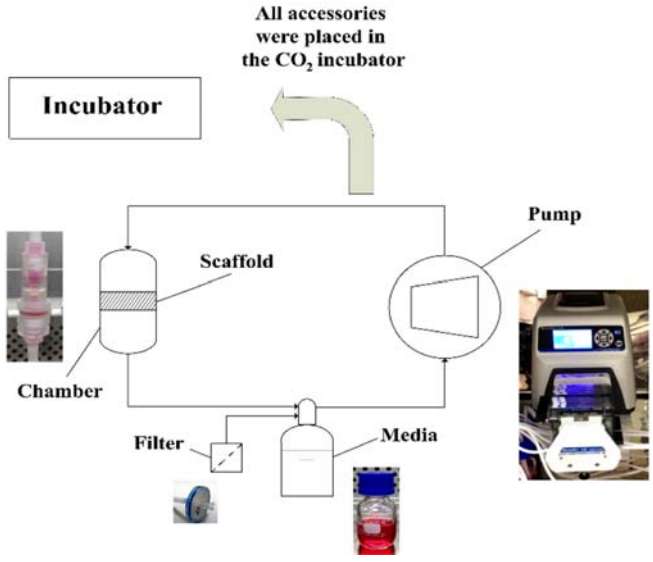

Figure 1. Schematic diagram of a flow perfusion bioreactor system.

2.7 Alamar Blue Assay

The Alamar blue assay was used in the assessment of the metabolic activity of cells. After 21-day culture period, the chondrocytes-seeded scaffolds were transferred into new 24-well plates which were later added with 1 ml of complete medium with resazurin (Sigma-Aldrich) dye (1:10; 0.1 mg/ml resazurin:complete medium) in each well. The whole plates were then incubated at 37 °C for 4 h in a humidified atmosphere of 5% CO_2. Afterwards, a 100 µl aliquot of the

solution from each well was transferred into a 96-well plate, to measure the fluorescence intensity of each sample by VICTOR™X4 multilabel plate reader (Perkin Elmer, Waltham, MA) with excitation at 530 nm and emission at 590 nm. Metabolically active (live) cells will convert non-fluorescent resazurin (blue) to highly fluorescent resorufin (pink color). The fluorescence intensity measured was, hence, directly proportional to the cell number and related to cell proliferation.

2.8 Dimethylmethylene Blue (DMMB) Assay

Total GAG content produced by chondrocytes cultured on each scaffold was evaluated by 1,9-dimethylmethylene blue (DMMB) assay. Briefly, after 21 days of culture period, each cell-seeded scaffold was lyophilized and digested with 1.5 mg/ml papain (Sigma-Aldrich) in papain buffer (5 mM L-Cysteine, 10 mM Na_2HPO_4, 5 mM EDTA) pH 7.5 at 60 °C for 18 h. The digested samples were centrifuged at 5000 rpm for 5 min. Aliquot 100 µl of each sample was taken and mixed with 100 µl of 2×DMMB dye solution in a 96 well-plate. The 10×DMMB solution was constituted of 80 mg DMMB (Sigma-Aldrich), 12.5 ml ethanol in 487.5 ml of formate buffer (4 g of sodium formate and 4 ml of formic acid in one liter of distilled water). The absorbance of each GAG-DMMB complex was measured at 525 nm in a VICTOR™X4 multilabel plate reader (Perkin Elmer, Waltham, MA) and converted into a GAG content by interpolation on the chondroitin-6-sulphate (Sigma-Aldrich) standard curve.

2.9 RNA Extraction and Real-time RT-PCR Analysis

Total RNA was extracted from each cell-seeded scaffold by TRIZOL reagent (Life Technologies, Carlsbad, CA) following manufacturer's instructions and then converted into first-strand complementary DNA (cDNA) using SuPrimeScript RT Premix (GeNet Bio, Korea). Real-time PCR reactions were carried out in Mx-3000P qPCR system (Agilent Technologies, USA) to determine the expression level of cartilage-specific genes, i.e., type II collagen and aggrecan, using 18S rRNA as an internal control. Porcine sequence-specific primers for type II collagen, aggrecan, and 18S rRNA used were obtained from Biodesign, Thailand, and presented in Table 1. The FastStart Universal SYBR Green master (Roche, USA) was used in the preparation of real-time PCR reactions in the condition of initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The relative amount of each gene was calculated using the comparative Ct method [24].

Table 1. Primer sequences used in real-time RT-PCR analysis.

Gene	Primer sequence
Aggrecan[a]	Forward 5'-TGC AGG TGA CCA TGG CC-3'
	Reverse 5'-CGG TAA TGG AAC ACA ACC CCT-3'
Type II collagen[b]	Forward 5'-GCTATGGAGATGACAACCTGGCTC-3'
	Reverse 5'-CACTTACCGGTGTGTTTCGTGCAG-3'
18S rRNA	Forward 5'-CCGCAGCTAGGAATAATGGA-3'
	Reverse 5'-AGTCGGCATCGTTTATGGTC-3'

[a] primer sequence of Aggrecanas described in Pei *et al.*

[b] primer sequence of Type II collagen described in Jefferies *et al.*

2.10 Histological Assays

After 21 days of culture period, all cell-seeded scaffolds were fixed with 4% paraformaldehyde at 4 °C for 2 h and then washed twice with PBS. The cell-fixed samples were dehydrated and then embedded in paraffin at 60 °C. The sample blocks were subsequently sectioned into 7 μm-thick slides. The sections were stained with hematoxylin-eosin (H&E) to observe cell infiltration and extracellular matrix (ECM) accumulation in the scaffolds, whereas the collagen content produced by chondrocytes was identified with Masson's trichrome staining, according to the Masson Trichrome Goldner (Bio-Optica, Milan, Italy) kit instruction, and then observed under an inverted microscope (Olympus IX71, Japan).

2.11 Immunofluorescence Analysis

The sections were deparaffinized, fixed with 4% paraformaldehyde, washed twice with PBS, and incubated with 0.1% Triton X-100 for 10 min to permeabilize cell membranes. The slides were subsequently washed twice with PBS prior to the addition of the blocking solution containing 2% bovine serum albumin. The slides were then incubated for 2 h. Next, the sections were washed three times with PBS and then incubated with primary antibody against type II collagen (ab3092, Abcam, Cambridge, MA) prepared at a 1:100 dilution at 4 °C overnight. Secondary antibody (AlexaFluor 647 rabbit anti-mouse IgG, ab150115, Abcam, Cambridge, MA) prepared at a 1:200 dilution was subsequently dropped on the PBS-washed slides before they were re-incubated at room temperature for 1 h. The cell nuclei present in the scaffolds were ultimately observed by a confocal laser scanning microscope (Olympus FLUOVIEW FV 10i, Japan) on the slides stained with DAPI (Molecular probes, Eugene, OR) prepared at a 1:1000 dilution and Prolong® Gold antifade reagent (Life Technologies, Eugene, OR)

2.12 Statistical Analysis

All the data were conducted at least in duplicate and presented as mean ± standard deviation (mean±SD). The statistical analysis was performed by Student's unpaired *t* test using SPSS software (SPSS for windows, version 11.5). Statistically significant results were considered at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Fabrication and Characterization of Fully Surface-modified PCL Scaffold

In the preparation of fully surface-modified PCL scaffold, PCL pellets were first alkaline hydrolyzed, to enhance the hydrophilicity of the entire PCL pellets prior to the fabrication into a 3D porous HPCL scaffold by a high pressure supercritical CO_2 technique. The surface of HPCL scaffold was further modified by a low-pressure oxygen plasma treatment, to augment the scaffold surface hydrophilicity. The surface morphology of both HPCL and plasma-treated HPCL scaffolds was characterized by SEM. As shown in Figure 2(a-b), the cross-sectional morphology of both scaffolds revealed similar interconnected porous structure with pore sizes in the range of 150-250 μm. The internal pore surfaces of the scaffolds were also micrographed, as demonstrated in Figure 2(c-d). The internal pore surface of the HPCL scaffold appeared relatively smooth, whereas that of the plasma-treated HPCL scaffold was roughed by plasma etching. This pore surface topography with nodule-like structure was raised from both crosslinking and scission of PCL chains upon the plasma treatment [25].

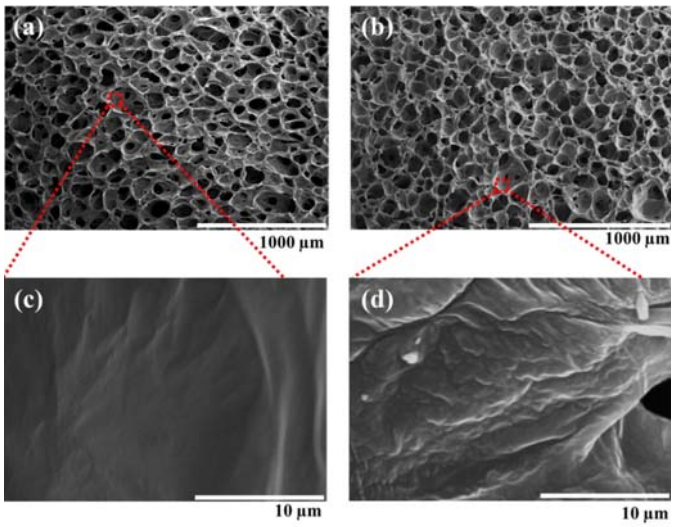

Figure 2. SEM images of cross-sections of the scaffolds modified by (a) alkaline hydrolysis solely (HPCL scaffold) and (b) alkaline hydrolysis and followed by plasma treatment (plasma-treated HPCL scaffold), and the internal pore surfaces of (c) HPCL and (d) plasma-treated HPCL scaffolds.

The surface hydrophilicity of PCL, HPCL, and plasma-treated HPCL scaffolds was comparatively evaluated by measuring the water contact angles on their surfaces by a sessile drop technique. The water contact angels measured as a function of time (0-60 s) observed on each scaffold are reported in Table 2. It was noted that the alkaline hydrolysis slightly improved the surface wettability of untreated PCL scaffold; all water contact angles measured on the HPCL scaffold were slightly lower than those on the untreated PCL scaffold. The hydrophilicity of HPCL scaffold was far enhanced after the plasma treatment. Considerable decreases in water contact angles on the plasma-treated HPCL scaffold were observed; moreover, the plasma-treated HPCL scaffold became absolutely wetted with a water contact angel of 0.00±0.00° at 15 s measure time, as shown in Figure 3. The drastically increased surface hydrophilicity of the HPCL scaffold was attributed to the formation of certain polar components, e.g., -C-O-, >C=O, and –COOH, on the material surface, upon the plasma treatment. Nevertheless, the alkaline hydrolysis of PCL pellets prior to the scaffold fabrication also accounted for the enhancement of surface hydrophilicity of the PCL scaffold.

Table 2. Water contact angles on different porous PCL scaffolds measured as a function of time.*

Sample	Water contact angle (X^o)		
	0 s	30 s	60 s
PCL scaffold	120.20±2.43	118.10±3.70	117.60±3.40
HPCL scaffold	108.80±6.15	106.50±6.83	100.50±10.91
Plasma-treated HPCL scaffold	42.55±1.06	0.00±0.00**	-

*n=6.

**The sample surface started to be completely wetted at 15 s.

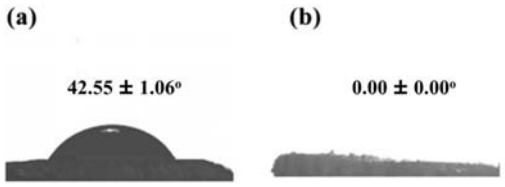

Figure 3. Water contact angles on the plasma-treated HPCL scaffold measured at: (a) 0 s and (b) 15 s.

The surface chemical composition of the scaffolds was also investigated by XPS measurement to complement the analysis of hydrophilicity of the surface-modified PCL scaffolds. The oxygen/carbon (O/C) atomic ratios found on the surfaces of different PCL scaffolds tested were directly compared. The XPS results were in good accordance with the water contact angle results stated above. The O/C atomic ratios detected on the surfaces of untreated PCL, HPCL, and plasma-treated HPCL scaffolds were 29.39±1.72%, 33.84±3.09%, and 50.28±2.02%, respectively, The O/C atomic ratio found on the surface of the plasma-treated HPCL scaffold was increased more noticeably, compared to that found on the surface of HPCL scaffold, indicating the presence of more oxygen-containing groups newly generated from the degradation of ester groups in PCL chains into -COOH and -OH groups. In fact, this degradation phenomenon was not only caused by the plasma treatment but also the alkaline hydrolysis. However, the plasma treatment outdid the alkaline hydrolysis.

3.2 Cell Proliferation

It has been demonstrated that the plasma-treated HPCL scaffold was prepared to possess rather homogeneous hydrophilic characteristics throughout the whole material. In the *in vitro* study under a static culture condition, it was proven that the highly hydrophilic outer surface of the scaffold was beneficial for initial cell attachment, whereas the porous inner material with increased surface hydrophilicity facilitated cell infiltration, resulting in the uniform cellular growth and distribution and ECM secretion and accumulation all over the porous scaffold [12].

The *in vitro* cultivation of porcine chondrocytes on the plasma-treated HPCL scaffold was comparatively conducted under static and dynamic conditions. The proliferation of chondrocytes after being cultured for 21 days was assessed by Alamar blue assay. As shown in Figure 4(a), the fluorescence intensities measured on the cell-seeded scaffolds incubated under both conditions were not different, suggesting that (live) cell numbers found under both culture conditions were about the same. This explicitly assured that the fully surface-modified PCL scaffold well supported the growth of chondrocytes.

3.3 GAG Content

The GAG secretion by porcine chondrocytes cultured on the plasma-treated HPCL scaffold was examined by using DMMB assay. Total GAG contents produced by the cells cultured for 21 days under static and dynamic conditions are comparatively presented in Figure 4(b). The amount of GAG secreted by the chondrocytes was raised from 28±11 μg/scaffold to 95±16 μg/scaffold when the culture environment was shifted from the static condition to the dynamic condition. The total GAG content significantly increased about 4-fold (p<0.05) when the chondrocytes were cultured in the in-house-built flow perfusion bioreactor at a constant flow rate of 0.1 ml/min which allowed fresh oxygen and nutrients to continuously flow through the cells being cultured on the porous scaffold. Under such

a more beneficial culture environment, all cells produced and deposited more GAG in the porous material, although the live cell number found on the scaffold in the bioreactor was about the same as that found on the scaffold in the well plates. Similar results were previously disclosed in the literature; chondrocytes cultured the scaffolds under dynamic environment at certain flow rates secreted great amounts of GAG [23, 26].

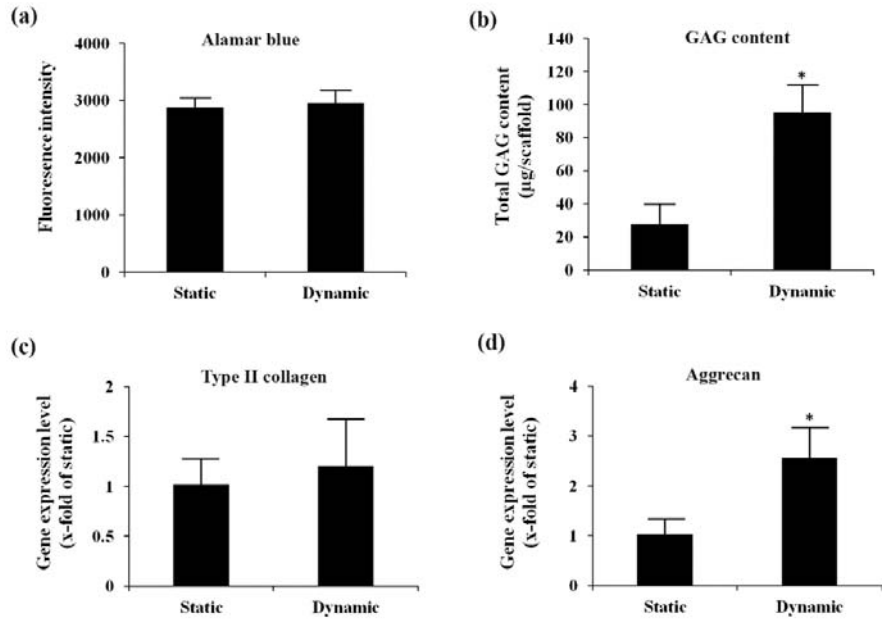

Figure 4. Biological responses of porcine chondrocytes cultured on the plasma-treated HPCL scaffold under static and dynamic conditions at 21-day culture period: (a) cell proliferation, (b) total GAG content, (c) type II collagen expression level, and (d) aggrecan expression level. The samples were tested in triplicate (n=3). Data are presented as means ± SD (*p<0.05).

3.4 Cartilage-specific Gene Expression

Type II collagen and aggrecan, two major specific components in the matrix of cartilaginous tissue, are essentially responsible for cartilage mechanical properties [27]. They are produced by chondrocytes and secreted into the extracellular matrix. The expression level of these two specific genes directly reflects the growth of chondrocytes and ECM formation. After 21 days of culture under static and dynamic environments, the mRNA expressions of type II collagen and aggrecan secreted from the chondrocytes cultured on the fully surface-modified PCL scaffold were determined using quantitative real-time RT-PCR. Both type II collagen and aggrecan expression levels measured are reported with respect to the values found under static condition, i.e., the average expression levels of type II collagen and aggrecan detected under static environment were set to 1, as shown in Figure 4(c-d). Though the mRNA level of type II collagen observed from the chondrocytes cultivated on the scaffold under dynamic condition was only slightly higher than that observed from the cells cultured in the well plate, a significantly greater aggrecan expression level (p<0.05) was detected from those cells cultured on the scaffold under dynamic

environment, compared to that detected from the cells cultured under static environment. These results vividly evidenced that the dynamic culture system could stimulate the synthetic potential of the porcine chondrocytes to express more cartilage-specific genes, especially aggrecan, owing to its advantages over the static culture system, in terms of oxygen, nutrient, and waste transfers.

3.5 Cell Histology and Immunofluorescence Staining

The cell number, cell distribution, and cartilage matrix production were histologically observed. Figure 5 reveals the histological appearances of H&E stained sections of the cell-seeded scaffold at a 21-day culture period under both static and dynamic environments. Both H&E-stained images revealed a similar homogeneous distribution of cells and cell infiltration pattern with a roughly equivalent cell numbers. The approximately equal number of live cells found in the stained-sections obtained from the static and dynamic culture conditions was in good accordance with the more or less equal fluorescence intensities read in the Alamar blue assay. The chondrocytes cultured in both environments infiltrated similarly downward through the porous scaffold. Hence, these histological results further confirmed the effectiveness of the fully surface-modified PCL scaffold in facilitating cell growth and infiltrations, regardless of the culture environment used. A tremendously higher amount of extracellular matrix was, however, produced by chondrocytes cultured on the scaffold in the flow perfusion bioreactor, as demonstrated in Figure 5(b). This result was also in good agreement with the results on the GAG secretion discussed previously. More ECM was anticipated to produce from cells being cultured under the environment that provided a better exchange of nutrients, oxygen, and wastes between cell membranes.

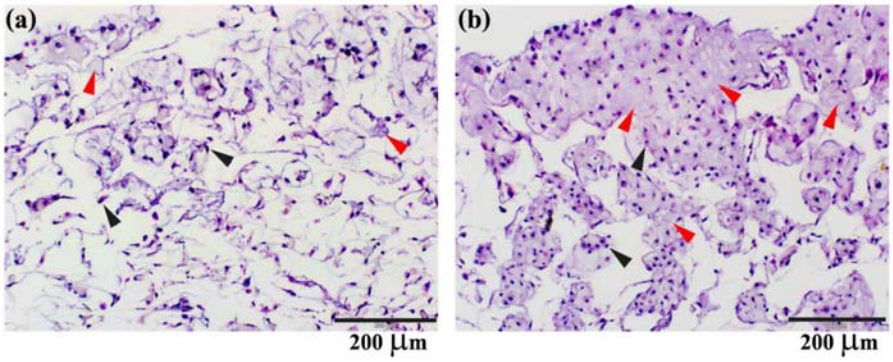

Figure 5. Histological appearances of Hematoxylin and Eosin (H&E) stained cross-sections of chondrocyte-seeded plasma-treated HPCL scaffold at a 21-day culture period under (a) static and (b) dynamic conditions. The black arrows indicate the chondrocyte nuclei, and the red arrows indicate the localization of extracellular matrix.

In generally, the collagen network helps preserve the extracellular matrix organization and provides proper tensile strength to cartilage function. In this study, Masson's trichrome staining was performed to observe the expression and distribution of collagen synthesized by the chondrocytes cultured on the scaffold under two different

environments. As revealed in Figure 6(b), an enormously greater amount of collagen fibrils was apparently visualized in the stained section obtained from the dynamic culture condition, suggesting that the chondrocytes functioned more efficiently in the presence of better transport of nutrients and waste products.

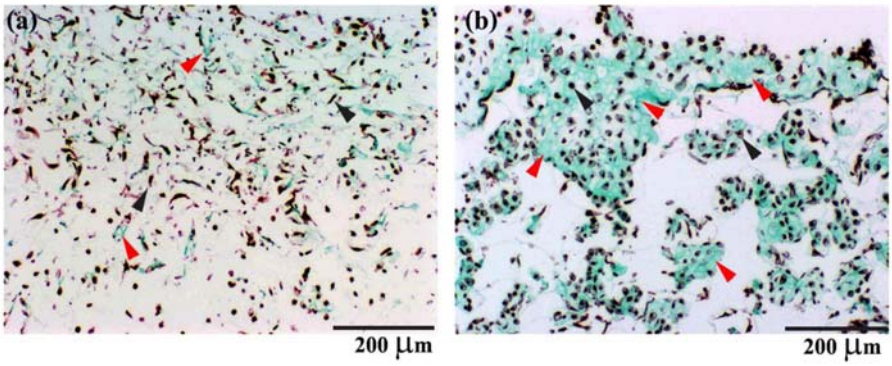

Figure 6. Histological appearances of Masson's trichrome stained cross-sections of chondrocyte-seeded plasma-treated HPCL scaffold at a 21-day culture period under (a) static and (b) dynamic conditions. The brown color, indicated by black arrows, represents the chondrocyte nuclei, and the green color, indicated by red arrows, represents the localization of collagen fibers.

The immunofluorescence assay was also performed to observe the secretion and deposition of type II collagen, a major collagen component in cartilaginous tissues. As seen in Figure 7(b), a significantly higher production and denser accumulation of type II collagen was found in the stained section obtained from the dynamic culture condition. The continuous circulation of culture medium through the scaffold in the bioreactor certainly enabled all the cells distributing throughout the scaffold to receive fresh and plenty of oxygen and nutrient supplies. As a consequence, the cartilage-specific substance produced by those cells was found in the higher amount.

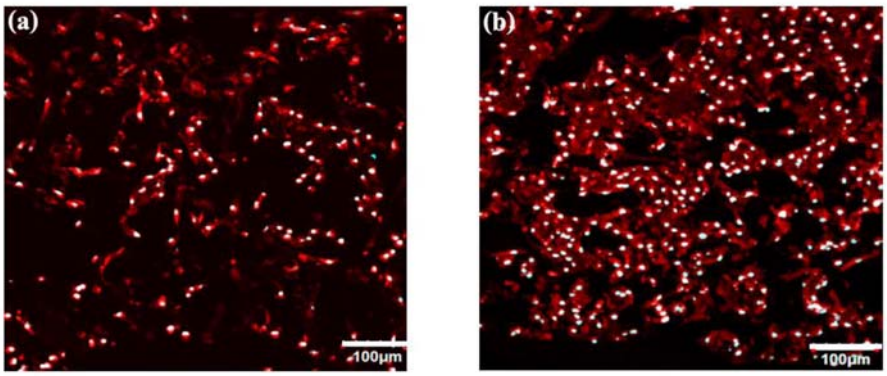

Figure 7. Immunofluorescence images of stained cross-sections of chondrocyte-seeded plasma-treated HPCL scaffold at a 21-day culture period under (a) static and (b) dynamic conditions. The red color represents type II collagen, and the light blue represents the chondrocyte nuclei.

CONCLUSIONS

The ability to induce cartilaginous tissue formation of cartilage cells cultured on the fully surface-modified PCL scaffold in two different culture environments was comparatively investigated. The dynamic culture performed in the flow perfusion bioreactor provided the more favorable exchange of nutrients and wastes between cell membranes, resulting in the greater chondrogenic gene expression levels and cartilaginous ECM production and accumulation by the porcine chondrocytes. Furthermore, the fully surface-modified PCL scaffold could well facilitate the growth and infiltration of the cells down through the whole scaffold, regardless of the culture conditions used, due to its homogeneously enhanced hydrophilicity of the entire scaffold. In summary, the use of the fully surface-modified PCL scaffold, together with the application of the flow perfusion bioreactor, remarkably stimulated the *in vitro* generation of cartilaginous extracellular matrix components.

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