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Improved Attachment and Proliferation of Porcine Articular Chondrocyte onto Hydroxyapatite Incorporated Poly(Lactic-co-Glycolic acid)

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Hydroxyapatite (HA) powders were incorporated in PLGA (75 : 25) films to investigate the effect of various HA contents in the composite film to the attachment and proliferation of porcine chondrocyte. The films were prepared by solvent-casting method. We observed the mechanical properties of the composite as well as chondrocyte attachment and proliferation of the prepared specimens. The 10 wt% HA content film showed two-fold higher ultimate tensile strength than control group. In surface characterization of the film, the surface of HA/PLGA composite films were more hydrophilic than that of control group ($p<0.05$). The cell attachment and cell proliferation of the HA/PLGA composite films (10 wt % HA) showed significantly higher than those of control group (1.44 times higher in attachment test, and 1.31 times higher at 6^{th}-day of culture in proliferation assaying, $p<0.05$). Based on these findings, 10 wt% of HA content in PLGA film was effective on improving mechanical properties as well as cultivating conditions for porcine chondrocyte.

Key words: Poly (lactic–co–glycolic acid), Hydroxyapatite incorporation, Chondrocyte, Attachment, Proliferation

INTRODUCTION

Loss of organs and damage of tissues lead to metabolic and structural changes that can cause significant disease and decrease the quality of life. It is well known that articular cartilage has a limited regeneration capacity.[1-3] Currently employed therapies for the treatments of articular cartilage loss or disease are unsatisfactory as they rely on joints prosthesis which offer structural replacement and limited functionality. Furthermore, artificial implants do not provide the lifelong solution for the patient. The purpose of tissue engineering is to improve the treatment for tissue and organ failure. Tissue engineering is to provide living biological/physiological substitutes that could replace tissue loss due to disease or accident. In comparison to artificial implants, ideal tissue engineered substitutes may offer a better long time performance due to its enhanced biocompatibility. The current treatment for articular cartilage repair suffers from certain limitation. The clinical need for improved methods for articular repair, there are many tissue engineering approaches to repair damaged articular cartilage *in vivo/vitro* from cells and films/scaffolds.[1,3-5,10] Among the synthesized polymers, poly(lactic acid), poly(glycolic acid), poly(ε-caprolactone), and their copolymers have been attracted in wide attention for their biodegradability and biocompatibility. However, the mechanical strength, toughness and elastic modulus of these polymers were lower than those of natural bone and cartilage tissue. Thus, for improving mechanical properties, inorganic fillers were introduced into biodegradable polymers to fabricate filler/polymer composites. Hydroxyapatite ($Ca_{10}(PO_4)_6OH$, Ca/P: 1.67) is used as a bone substitute, non-toxic, which can offer stability with host bone. In this manner, poly(lactic-co-glycolic acid (PLGA)/HA composite is expected to offer improved biocompatibility from biological and mechanical view point.[7,11,13] Practically, regarding the PLGA/HA composites were reported mainly on mechanical properties and bone cells.[7-13]

In this study, we fabricated PLGA (75:25)/Hydroxyapatite composite films with various HA contents to investigate adequate HA content ratio that might improve cell attachment and proliferation for cartilage reconstruction as well as enhancement of mechanical properties.

MATERIALS AND METHODS

Preparation of PLGA and HA Composite

PLGA (M_w = 113 kD) was purchased from LakeShore-Biomaterials. (Brookwood Pharmaceuticals, Inc., Birmingham, Alabama, U.S.A.) HA powder was obtained from Sigma-

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Aldrich. (Sigma-Aldrich, Inc.) PLGA/HA composite films were fabricated by solvent-casting method. PLGA powder (with HA 5, 10 and 15 wt%) was dissolved in chloroform, the solution was cast into a mold. The solvent was evaporated overnight at ambient atmosphere, and vacuum freeze-dried during 48 hrs. PLGA film without HA were also fabricated by the solvent-casting, for control group.

Physical Characterization oF Composite Films

Static contact angle measurement of the PLGA/HA composite specimens was performed and characterized by contact angle analyzer (Phoenix 150, SEO Ltd., Suwon, Gyeonggi-do, Korea).

Morphology study of the PLGA/HA composite film had done by the FE-SEM (HITACHI S-4300DSE., Japan) at 5 kV.

For mechanical property of the prepared specimens, tensile test was performed with a Micro-Load System (R & B INC. Korea). The samples were cut (15×10 mm) from PLGA/HA films with 0.14±0.02 mm (n=5) thickness. Measurement was carried out at a cross-head speed of 2 mm/min with 5 kgf load.[6)]

The specimens were elongated until failure occurred. The elastic modulus was determined from the slope in the initial elastic portion of the stress-strain diagram.

Chondrocyte Isolation and Culture

Full-thickness articular cartilage was obtained from the joint of porcine rear leg and chipped into small pieces at the size of 0.5×0.5×0.2 mm. Chondrocytes were isolated from the cartilage by trypsin treatment for 10 min, followed by type II collagenase (GIBCO) (2 mg/ml) digestion for 8-12 hrs, and filtered by a nylon sieve (cell strainer, Falcon) with an 80 μm pore size. The cells were rinsed three times with Hank's balanced salt solution (HBSS) by centrifugation at 1000 rpm for 3 min. Then, cultured in cell culture flasks in a density of $2.0\times10^4/cm^2$ with Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS (GIBCO), 120 mg/L penicillin and 200 mg/L streptomycin, in a humidified incubator at 37°C and 5% CO_2. The medium was changed every 3 days.

Cellular Attachment and Proliferation

Cellular attachment and proliferation on PLGA/HA composite films at different time intervals were investigated. The films were cut (φ =12 mm) and placed into 24-well plates (Falcon), washed three times with PBS. The 2nd-passage chondrocytes were used for cell attachment test with density of 1.3×10^5 cells/film. The plates were incubated at 37°C and 5% CO_2 for 4hrs. After that, the medium was added by 1 ml for next another 4 hrs incubating. The attached cells on the film were measured by Cell Counting Kit (CCK-8, Dogindo Lab, Kumamoto, Japan). After adding 10 μl CCK-8 solution to 24-well plate, the CCK-8 solution which reacted with cells for 4 hrs on the film had changed in color from pink to orange. The absorbance of the changed orange color solution was measured at 450 nm using a micro-plate reader (synergy HT, BIO-TEK) to determine the amount of attached cells.

For cell proliferation assaying, 1.3×10^4 (cells/film) initial cell density of the chondrocyte was cultivated in a humidified incubator which containing 5% CO_2 at 37°C. The proliferated cells were measured at 1st, 3rd and 6th day of culture.

Cell Morphology Analysis

The specimens of 6th-day harvest were fixed by 3.0% formaldehyde solution for cell morphology and distribution analysis. The cells were stained with hematoxylin and eosin (H&E) and observed by optical microscope (BX51, OLYMPUS).

Statistical Analyses

The results were expressed as mean ± standard error (S.E.) for all comparisons. The one-way analysis of variance (ANOVA) method was used to determine the significance of difference among multiple times. A difference was considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Physical Properties of PLGA/HA Composite

The contact angles of all PLGA/HA composite groups were decreased as compared with control group (Table 1). The PLGA/HA composite specimens possess more hydrophilic surface of the film than PLGA film (control) ($p < 0.05$). This may due to the hydrophilicity of HA.

The SEM observation had been done in order to investigate the dispersion homogeneity of HA particles in the PLGA matrix. In the 15 wt% HA composite, many clots colony were observed throughout the specimen (Figure 1 (c)), and a fine fracture surface was detected in the HA clots (Figure 1 (d),

Table 1. Properties of PLGA/HA composites determined by contact angle and tensile tests (n=5) (*$p<0.05$)

	Contact angle	Ultimate tensile strength (MPa)	Young's modulus (MPa)
PLGA	68	1.89±0.4	3.09±0.30
PLGA/5%	61.75 ±4.06	3.4 ±0.3	7.2 ±0.28
PLGA/10%	58.125±2.1	3.9 ±0.37*	10.4 ±0.62*
PLGA/15%	56 ±1.51*	1.77±0.1	8.5 ±0.53

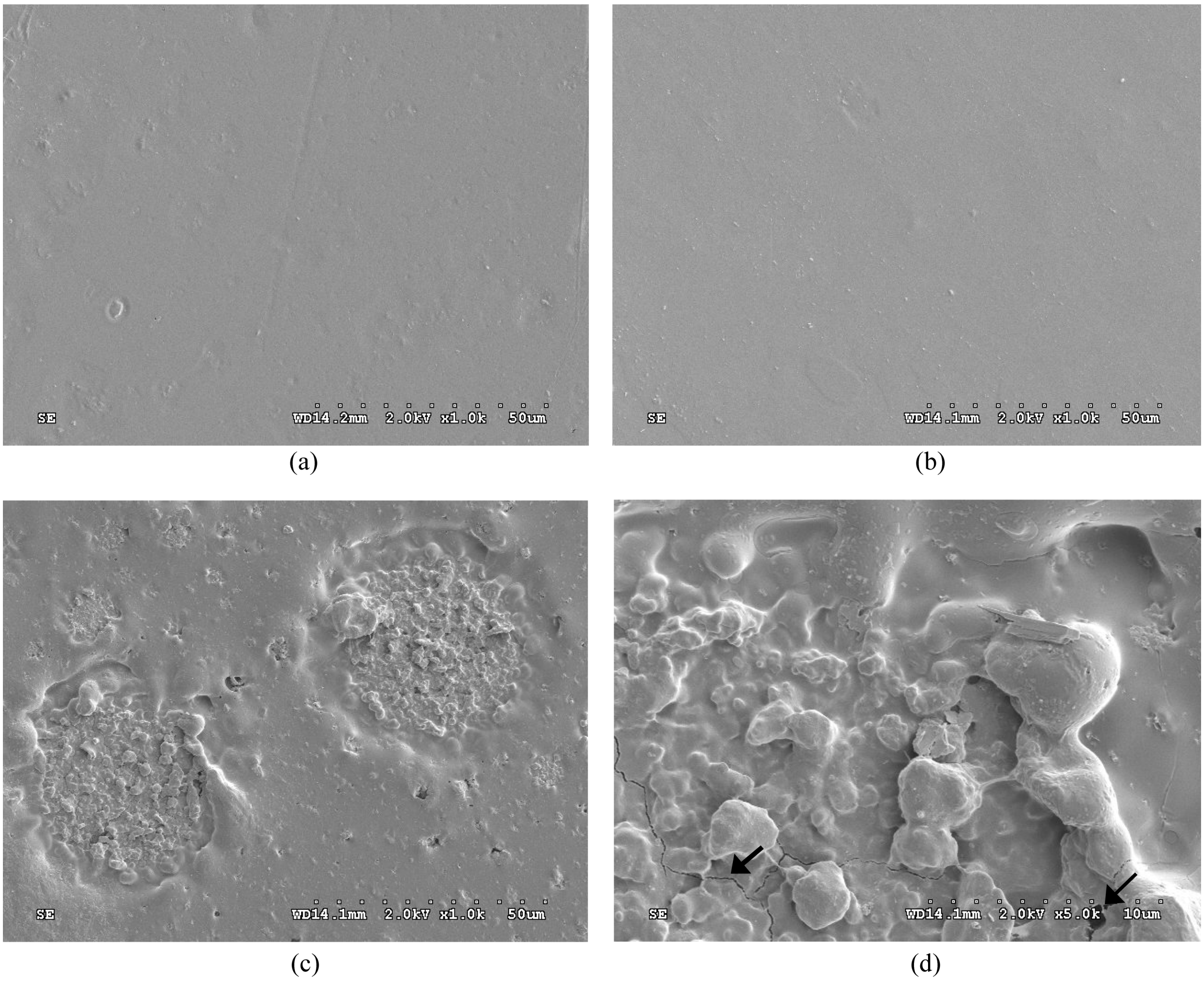

Figure 1. SEM image PLGA/HA composite films (a) PLGA/HA5 wt%; (b) PLGA/HA10 wt%; PLGA/HA 15 wt% (c) x 1000 and (d) x 5000, arrows : cracks in HA powder clot.

two arrows). On the other hand, for 5 and 10 wt% HA composite, no clots were found in the specimens (Figure 1 (a), (b)). Moreover, it had shown that fine HA particles were spread throughout the 5 and 10 wt% HA composite. This meant that mixed HA powders were dispersed on the film very evenly.

The ultimate tensile strength and Young's modulus of HA content composites were given in Table 1. Compared the PLGA/HA composites with control group, it showed increased tensile strength and young's modulus at the HA particle content of 5 and 10 wt%. Especially, for 10 wt% HA composite, ultimate tensile strength was 2 times higher (3.9 ± 0.37MPa) and Young's modulus was 3.3 times (10.4 ± 0.62 MPa) higher than control group. Petricca *et al*. reported that 10 wt% HA composite showed a large ultimate tensile strength (9.4 ± 0.246 MPa). Beyond 10 wt%, ultimate tensile strength was decreased.[14] This is similar trend compared with our results. As expected, the 15 wt% of HA composite showed high young's modulus (8.5 ± 0.53 MPa), but significantly decreased in tensile strength (1.77 ± 0.1 MPa). The HA particles incorporation augment Young's modulus of the composite, on the other side, it made composite tough. Thereby, the slope of elastic curve was very stiff and the composite was hard, so the specimen was broken quickly. This may be caused by weak bonding between HA and PLGA polymer interface[14] (Figure 1 (d)). The 5 and 10 wt% HA composite films offered mechanical stability for cell attachment and proliferation.[11]

Chondrocyte Behavior Onto PLGA/HA Composite

The result of cell attachment on PLGA and PLGA/HA composite films were shown in Figure 2 (a). Compared with each group, PLGA/HA (10 wt%) composite showed 1.44 times more cells were attached on film than control. ($p<0.05$) It might be expected to increase the chances of cells to make contact with HA, which was believed to enhance cell's adhesion and proliferation.[6,12,14] Cell attachment is considered as an important thing because it influences cell proliferation.

Figure 2 (b) showed cell proliferation assaying of the speci-

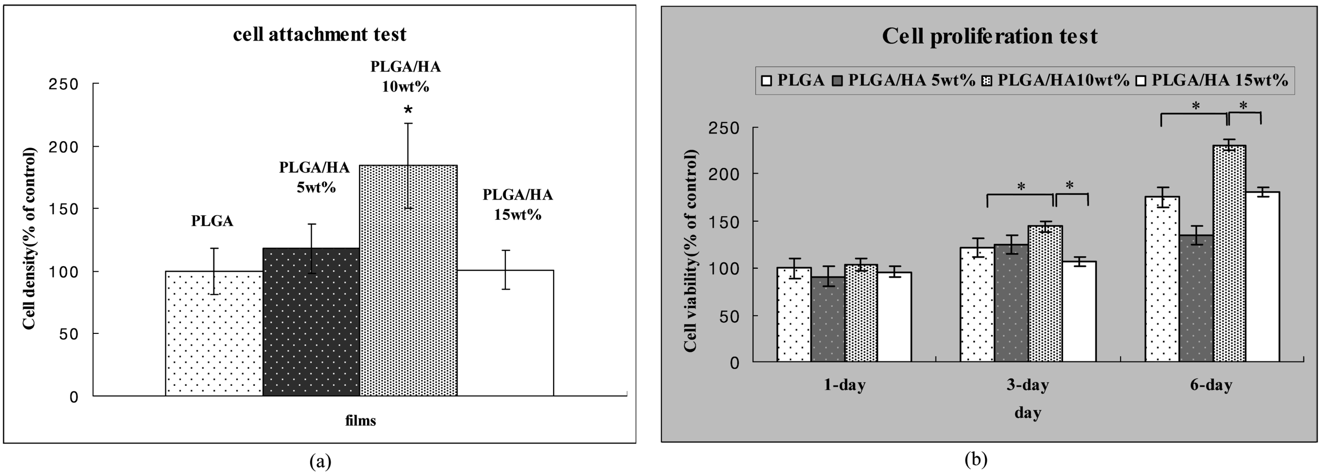

Figure 2. Effect of hydroxyapatite on chondrocytes attachment (a) and proliferation (b) (n=5), (*$p<0.05$).

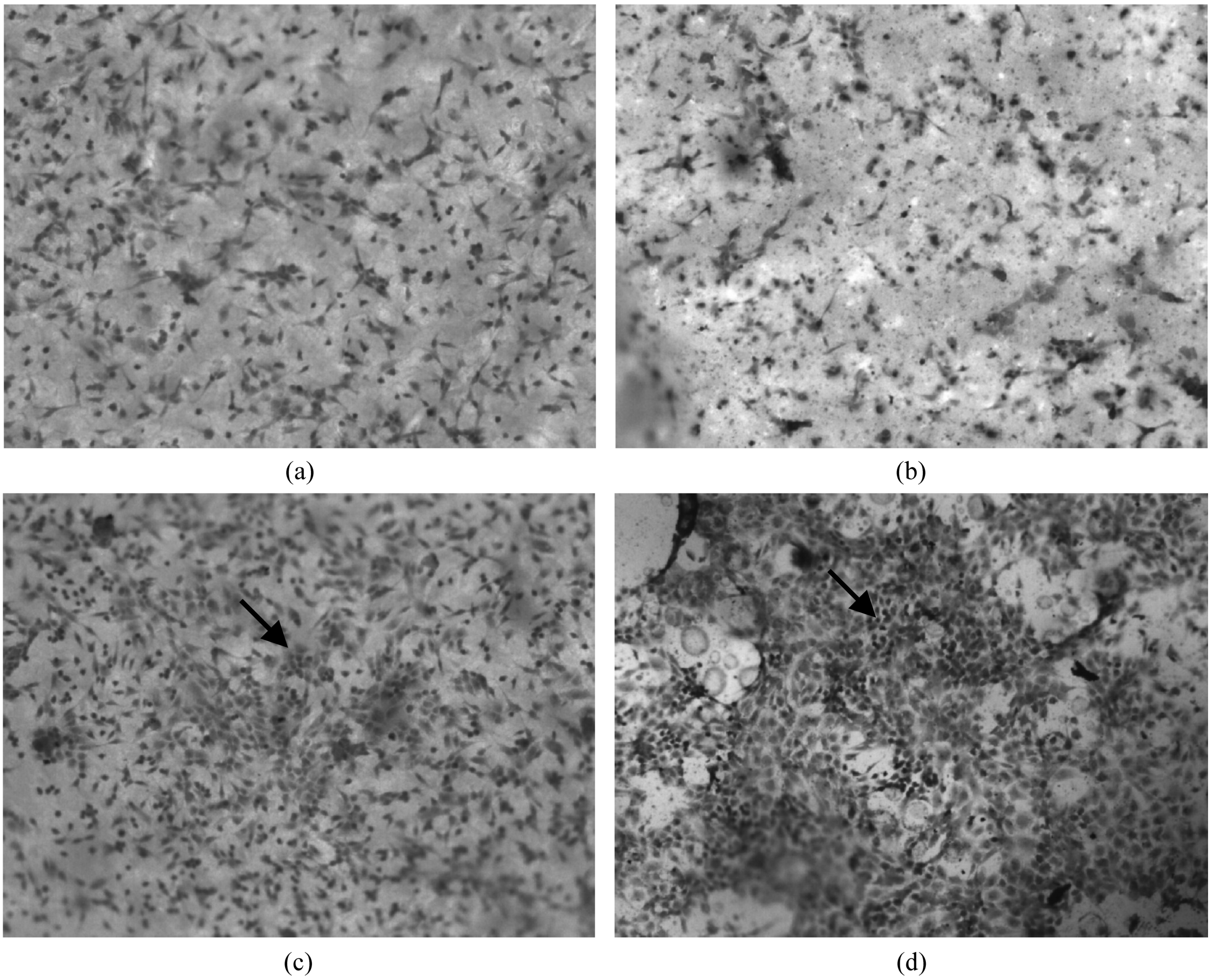

Figure 3. H&E image of PLGA/HA composite film at 6th-day culture in proliferation test PLGA (a), PLGA/HA 5%, (b) PLGA/HA 10% (c) and PLGA/HA 15% film (d); Arrows of (c) and (d) showed that morphology of chondrocytes was rounder than control group. (magnification : x50).

mens. Cell quantity of PLGA/HA (10 wt%) composite showed 1.3 times higher than control group at 6th-day of culture. It showed significantly improved proliferation in statistical analysis. ($p<0.05$) The 15 wt% of HA group, non-homogeneously dispersity of HA particles caused decrease tensile strength, degradation and destruction of the film and seemed to give damage on attached and proliferated cells.[11] Strangely, in the 5 wt% HA composite, less cells were proliferated than control at 6^{th}-days. It may the cells' chance of contact with HA was lower than 10 wt%; it was not enough to effective on cell proliferation.

The HA within the polymer matrix improving cell growth, especially, 10 wt% amount of HA enhanced cell attachment and proliferation as shown in Figure 2.

Histology of Chondrocyte-populated PLGA/HA Composites

The H&E assay for chondrocytes throughout the various composite films was presented in Figure 3. The morphology of chondrocytes on 10 and 15 wt% HA composite groups were rounder than that of control and 5 wt% HA composite groups. Compared 10 wt% HA composites with 15 wt% HA composites (Figure 3 (c) and (d)), there were no dramatic differences in the histological appearance. However, much more chondrocytes were distributed in the 10 and 15 wt% HA composites (Figure 3 (c) and (d)) than the other groups. A homogeneous distribution of attached cells is important and necessary for the development of tissue engineering.[13] The 10 and 15 wt% HA composite films were effective in chondrocytes proliferation with an evenly distribution in this study.

CONCLUSIONS

The PLGA/HA composite material enhanced chondrocyte adhesion and proliferation *in vitro* study. Also, the composite showed improved mechanical property as well as surface property of the film. The enhancement of cell attachment and proliferation may result from the exposed/embedded bioactive HA particles on PLGA film surface which were biocompatible and similar to the mineral phase of native bone tissue in chemical and structural point of view.[12] Especially, 10 wt% HA composite film showed a maximum cell attachment and proliferation among the groups.

Conclusively, the PLGA/HA 10 wt% composite film could enhance the cartilage regeneration efficiently for chondrocyte attachment and proliferation for the treatment of cartilage defect, as compared with PLGA/various content of HA films.

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