3-dimensional composite scaffolds consisting of apatite-PLGA-atelocollagen for bone tissue engineering

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We fabricated 3-dimensional scaffolds consisting of biodegradable poly(D, L-lactide-co-glycolic acid)(PLGA)(75/25) with hydroxyapatite particles containing atelocollagen (aAC). The aim of this study was to evaluate this new type of scaffold in regard to its basic properties and biocompatibility. Characterization of the obtained scaffolds was performed to know the porosity, shrinkage, diametral tensile strength, and biocompatibility. Composite scaffolds made of PLGA with hydroxyapatite particles containing atelocollagen (PL-aAC) showed a greater strength and stability than PLGA scaffolds. PL-aAC also exhibited superior performance in terms of cell attachment and proliferation as compared to PLGA, while histological findings showed that PL-aAC had an excellent response toward soft tissues. Our results strongly suggest that PL-aAC is more useful for cell transplantation as compared to PLGA for bone tissue engineering.

Keywords: PLGA, Apatite, Atelocollagen, Scaffold, Bone tissue engineering

INTRODUCTION

Recently, poly(D,L-lactic-co-glycolic acid)(PLGA) has been investigated as a scaffold material for applications in bone tissue engineering. Although PLGA is biodegradable and hydrophobic, porous scaffolds fabricated with PLGA float in cell culture medium, while they also have limited ability to promote cell attachment and proliferation¹⁾. Moreover, it was reported that PLGA scaffolds induced a slight inflammatory response after implantation *in vivo*²⁾. To improve these drawbacks of PLGA only, we fabricated 3-dimensional (3D) scaffolds consisting of PLGA with apatite cement (AC) particles containing atelocollagen for use as scaffolds in bone tissue engineering.

AC is a bioactive cement that transforms to hydroxyapatite (HAp) when exposed to moisture³⁾. Various types of AC have been developed, in attempts to improve the properties of AC^{4,5)}. In the previous study, we fabricated AC containing atelocollagen (aAC) and evaluated its feasibility *in vivo*⁶⁾. As a result, it was found aAC showed an excellent tissue response when aAC was implanted subcutaneously in rats. Notably, collagen accelerates cell attachment, proliferation, and osteoconduction when used as a coating material on calcium phosphate ceramics, such as HAp and tricalcium phosphate. Furthermore, collagen, a primary component of the organic substrate comprising bone, is known to play important roles in the formation and mechanical properties of bone. Based on its physical and biological properties, it is often used in preparation of resorbable biomaterials⁷⁾. Although an immunogenic reaction is a serious problem when collagen is applied in biomaterials,

atelocollagen is more preferable for biomaterial use as compared to other kinds, because it has the lowest level of antigenicity⁸⁾.

In the present study, we developed PLGA with aAC composite scaffolds (PL-aAC) using a solvent casting/particulate leaching method. The objective of our investigation was to fabricate novel 3D scaffolds and evaluate their biocompatibility, mechanical strength, shrinkage, and ability to promote cell attachment and proliferation.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-co-glycolic acid)(PLGA)(75/25); corefront corporation, Tokyo, Japan) produced with a copolymer mass ratio of 75:25 from lactide and glycolide using stannous octoate as the initiator, and an inherent viscosity of 0.68 dL/g at -10°C in CHCl₃, was prepared. The other materials used in this study were tetracalcium phosphate (TTCP; Taihei Chemical Industrial Co., Osaka, Japan), dicalcium phosphate anhydrous (DCPA; J.T. Baker, N.J., USA) and dimethyl sulfoxide (DMSO; Sigma Chemical Company, MO, USA). Sugar particles (Mitsui Sugar Co., Ltd, Tokyo, Japan) were passed through a 1 mm sieve.

Preparation of aAC powder

The powder phase of AC was prepared as previously described⁹⁾. In brief, an equimolar mixture of TTCP and DCPA was mixed using a speed mill (SK-M2, Kyoritsuriko, Tokyo, Japan), then sterilized by exposure to 20 kGy of gamma radiation and kept in a vacuum

desiccator set at 60°C until use. For the preparation of aAC, 2% atelocollagen gel (KOKEN Co., Ltd, Tokyo, Japan) was used as the liquid phase⁹. The powder and liquid phases were mixed at a P/L ratio of 2.0, then the obtained paste was allowed to harden at room temperature for 24 h. Thereafter, it was crushed into powder and passed through a 100 µm sieve. The atelocollagen content of aAC (1 g) was 0.1 mg. The resultant powder was poured and mixed with PLGA solution as follows.

Preparation of PL-aAC composite scaffold

PL-aAC composite scaffolds were fabricated using the solvent casting/particulate leaching method¹⁰. In brief, PLGA(75/25) was dissolved in DMSO according to the concentration of 10%(w/w). PLGA solution was mixed with sugar granules and aAC powder at a PLGA:aAC of 1:1 [PL-aAC(1)] and 1:2 [PL-aAC(2)]. At the same time, a PLGA solution with no aAC was mixed with sugar granules of 1 mm [PL]. Then, all of the dispersion was cast into a mold and frozen immediately at -18°C. The frozen sample was soaked into deionized distilled water (ddH₂O) and the sugar granules were leached out of the PLGA-aAC mixture. Subsequently, the produced scaffolds were dried for 2 days. In this manner, 3.0×3.0×5.0 cm scaffolds were produced. The samples were cut into cubes for each test. After sectioning, the atelocollagen content of PL-aAC(1) and PL-aAC(2) was 1.10×10^{-3} and 2.19×10^{-3} mg, respectively.

Scanning electron microscopy (SEM)

The surface and cross-sectional morphologies of porous PLGA and PL-aAC were observed using a scanning electron microscope (SEM, JSM-5300, JEOL, Tokyo, Japan) at an acceleration voltage of 15 kV. After incubation, cell seeded scaffolds were quickly rinsed with 0.1 mol·L⁻¹ cacodylate buffer 2 times, then fixed in Karnovsky's fixative at room temperature and placed into a 4°C bath for no less than 2 h. Next, they were dehydrated through a graded ethanol series, critical point dried with liquid CO₂, and sputter-coated with 10 nm of gold under a vacuum condition.

Pore size and porosity analysis

The mean pore diameter was evaluated using the SEM images of cell-free scaffolds. Linear measurements from the most distant points of the pore openings were recorded ($n=10/\text{scaffold}$)¹¹. Apparent porosity was determined using a method similar to that reported by Xinhua *et al.*¹². Briefly, a cube sample (5×5×7 mm) was cut out of a scaffold sample, then the volume was calculated from the measured dimensions, and the mass was determined with an analytical balance. Five samples of each scaffold were measured to calculate average density. Apparent porosity was calculated from the scaffold density (P) and the polymer skeletal density (P₀) using the following equation:

$$\text{Porosity (\%)} = (1 - P/P_0) \times 100$$

X-ray diffraction (XRD) analysis

The compositional change in PL-aAC upon setting or gamma irradiation was analyzed using powder X-ray diffraction (XRD; Rint 2000, Rigaku, Tokyo, Japan). Measurements were performed with a vertically mounted diffractometer system with Ni filtered CuKα radiation (0.154 nm) generated at 30 kV and 16 mA. The samples were scanned from 3 to 60° in 2θ (where θ is the Bragg angle) to determine the reaction product in continuous mode.

Mechanical strength measurement

The mechanical strength of PLGA and PL-aAC was evaluated in terms of diametral tensile strength (DTS). The column shaped samples were cut at a diameter of 8.5 mm and height of 10 mm. Testing was performed under four conditions: dry, and wet /37°C /2 weeks, 4 weeks, and 8 weeks. The samples were crushed at a cross-head speed of 1 mm min⁻¹ using a universal testing machine (AGS-500A, Shimadzu, Kyoto, Japan).

Shrinkage test

The samples were cut into 5×5×7 mm cubes for the shrinkage test. The mass of each scaffold was measured before and after incubation in α-MEM at 37°C for 2, 4 and 8 weeks, respectively. The % decrease in mass was calculated from the following equation¹³.

$$\text{Mass loss (\%)} = 100 \times (M_0/M_t)/M_0$$

Where M with subscript 0 and t indicates the mass at the immersion time of 0 and t, respectively. All values are presented as averages of the samples.

Initial cell attachment and proliferation of MC3T3-E1 cells

In this study, MC3T3-E1 cells were used. The culture medium was α-MEM (Dainihonsei-yaku, Osaka, Japan) supplemented with 10% fetal bovine serum (Whittaker Bioproducts Inc., Walkerville, MO, USA). Each scaffold was placed in each well of a 48-well plate (Corning, NY, USA). The prepared porous scaffolds were cut into 5×5×7 mm cubes and prewetted in phosphate-buffered saline (PBS) for 2 h. MC3T3-E1 cells (1×10^4) were resuspended in 100 µL of media, then the concentrated cell suspensions were pipetted onto scaffolds and incubated in an atmosphere containing 5% CO₂ at 37°C. After 2 h, 1 mL of medium was added to the 48-well plate (Corning) and the medium was changed every 3 days. After culturing for 5 h, 1 week, and 2 weeks, the number of cells was counted.

Histological preparations

For the animal experiments, the housing care and experimental protocol were approved by the Animal Care and Use Committee of the University of Tokushima School of Dentistry. Eight-week-old male Wister rats were obtained commercially (Charles River Laboratories, Inc, Wilmington, MA, USA) and fed standard pellets and water freely, then used for a biocompatibility study of

Fig. 1 SEM images of (I) PLGA, (II) PL-aAC(1), and (III) PL-aAC(2) (Scale bars=500 μm) .

the scaffolds without cells. PLGA, PL-aAC(1), and PL-aAC(2) samples were cut into 5×5×7 mm cubes and surgically implanted into subcutaneous tissue in the abdomen of 6 rats. The soft tissues containing scaffolds were removed from 3 rats each at 3 days and 3 weeks after surgery, then fixed in 10% neutral-buffered formalin and embedded in resin (OsteoResin; Wako). Finally, they were sliced into 5- μm -thick serial longitudinal sections, stained with hematoxylin-eosin, and subjected to light microscopic examination.

RESULTS

Figure 1 shows SEM images of the surface and cross-sectional morphologies of porous (I)PLGA, (II)PL-aAC(1), and (III)PL-aAC(2). PLGA and PL-aAC had the highly interconnected macroporous structure of representative scaffold. When more aAC particles were added, the small pores decreased, and consequently, the level of interconnection decreased. Figure 2 shows the pore size and porosity of a representative scaffold. The line graph indicates the porosity, and the bar graph shows the pore size. There were no significant difference among PLGA, PL-aAC(1), and PL-aAC(2). Figure 3 presents comparisons of XRD patterns for PLGA, PL-aAC(1), and PL-aAC(2) with those for the powder phase of PLGA and aAC. The peaks assigned to HAp (25.9° and 31.8° in 2 θ) were detected in PL-aAC(1) and PL-aAC(2). The mechanical strength of each scaffold is shown in Fig. 4. The DTS of PLGA was significantly lower as compared with that of PL-aAC(1) and PL-aAC(2) under all testing conditions, while higher aAC ratios produced better mechanical properties under all conditions. Figure 5 shows the shrinkage test of PL-aAC(2), PL-aAC(1), and

Fig. 2 Pore size and porosity of the scaffolds. SEM images of cell-free scaffolds were evaluated to determine the mean pore diameter. Linear measurements from the most distant points of pore openings were recorded ($n=10/\text{scaffold}$). Descriptive statistics were calculated and comparisons of the mean pore diameters among the scaffolds were evaluated using analysis of variance ($\alpha=0.01$).

PLGA before and after incubation in α -MEM for 2, 4 and 8 weeks, respectively. As a result, the mass reduction of PLGA was larger than of PL-aAC(1) and PL-aAC(2) at the same time periods, while the addition of aAC particles to the scaffolds resulted in a significant mass reduction. Figure 6 shows the initial attachment and proliferation of MC3T3-E1 cells on scaffolds. The initial attachment and proliferation of MC3T3-E1 cells to PL-aAC(1) and PL-aAC(2) were significantly greater than with PLGA. Specifically, the number of cells proliferating in PL-aAC(1) and PL-aAC(2) was significantly increased as compared to that in PLGA at 2 weeks. Addition of aAC particles to the scaffolds resulted in a significant increase in initial cell attachment and proliferation. Figure 7

Fig. 3 Powder X-ray diffraction patterns of (I) PL-aAC(2), (II) PL-aAC(1), (III) PLGA, and (IV) aAC. The powder phase of calcium phosphate cement and poorly crystallized aAC are shown for comparison.

Fig. 4 DTS value of PLGA, PL-aAC(1), and PL-aAC(2) under three conditions: dry, wet /37°C /1 week and 2 weeks.

shows SEM images of MC3T3-E1 cells cultured for 2 weeks on PLGA, PL-aAC(1), and PL-aAC(2). The shape of the cells expanded on the surface was nearly the same with all of the scaffolds. Figure 8 summarizes the histological appearances of soft tissues associated with PLGA and PL-aAC(2). PL-aAC(2) at 3 days after implantation showed slight inflammation consisting of a smaller number of lymphocytes, plasma cells, and macrophages as compared to PLGA. On the other hand, the inflammatory response of tissues associated with PL-aAC(2) was resolved after 3 weeks. These results indicate that addition of aAC to PLGA resulted in an excellent response in associated soft tissues as compared with PLGA alone.

DISCUSSION

Scaffolds play an essential role in supporting bone regeneration, as their 3D structure provides physical support and acts as a substrate to regulate cell growth, adhesion, and differentiation¹⁴. However, they also have some disadvantages. The degradation products reduce the local pH value, which in turn, may accelerate the

Fig. 5 Shrinkage test results with PL-aAC(2), PL-aAC(1), and PLGA before and after incubation in α -MEM. The results are shown as the mean $\pm$ standard deviation ($n=5$). * and bars indicate significant difference as compared with PLGA and others, respectively.

Fig. 6 Initial attachment and proliferation of MC3T3-E1 cells on scaffolds. The numbers of MC3T3-E1 cells on scaffolds were determined after 5 h, 1 and 2 weeks of culturing. The results are shown as the mean $\pm$ standard deviation ($n=5$). * and bars indicate a significant difference as compared with PLGA and others, respectively.

polyester degradation rate and induce an inflammatory reaction. In addition, the mechanical properties of PLGA are relatively low, which limits its use in bone tissue regeneration, especially at the *in vivo* implant site¹⁵. Also, PLGA has a low level of surface wettability due to its composition of non-charged elements. Such hydrophobic surfaces are unfavorable to osteogenic cells, as they show a lower proliferative and higher apoptotic rate on hydrophobic surfaces as compared to hydrophilic surfaces^{15,16}. Finally, PLGA has a bioinert surface that lacks bioactive functions for bone formation, therefore evoking minimal tissue responses¹⁷.

HAp has been investigated as a bone replacement material since it mimics natural bone mineral and development of biodegradable polymer/bioceramic composites could be a solution to the problems noted above. The incorporation of synthetic HAp into PLGA is expected to result in a promising composite scaffold for bone tissue engineering, as improved hydrophilic and

Fig. 7 SEM images of MC3T3-E1 cells cultured for 2 weeks on (I) PLGA, (II) PL-aAC(1), and (III) PL-aAC(2) (Scale bars=10 μm) .

Fig. 8 Photomicrographs of subcutaneous tissue surrounding (I) PLGA and (II) PL-aAC(2) scaffolds at 3 days after surgery, and (III) PLGA and (IV) PL-aAC(2) 3 weeks after surgery. (hematoxylin-eosin stain) (Scale bars=250 μm).

osteoconductive properties may be provided by HAp, while the acidic degradation byproducts from PLGA may be buffered, resulting in better mechanical properties^{18,19}.

In the present study, we used the solvent casting/particulate leaching method to fabricate highly porous PL-aAC composite scaffolds, which resulted in a highly

interconnected macroporous structure. XRD patterns in PL-aAC confirmed the presence of aAC regardless of the presence or absence of PLGA. Also, PLGA acted as a matrix and aAC particles acted as fillers in the PLGA network. Therefore, it was confirmed that a higher PL-aAC ratio did produce superior mechanical properties.

Scaffolds used for bone tissue engineering should maintain their structural integrity during cell culture. However, it was reported that PLGA undergoes bulk degradation by simple hydrolysis into lactic and glycolic acids²⁰. Moreover, the acidic degradation products reduced the local pH and accelerated the degradation rate²¹. It was also reported that the pH of PLGA/HAP composite is more stable than that of PLGA and HAP scaffolds¹³. In the present study, PL-aAC showed a decreased level of acidic degradation by products from PLGA during incubation, and maintained its structure and pores for new bone formation, thus the shrinkage of PL-aAC was superior to that of PLGA. Cell attachment and proliferation are affected by the hydrophilic property, structure, and chemical composition of the scaffold surface. Also, the amount of cell adhesive proteins in aAC, such as fibronectin and vitronectin, subsequently dictate cell adhesion behavior^{22,23}, because most cells attach to appropriately modified surfaces far better than to hydrophobic surfaces, including those with fewer proteins²⁴⁻²⁸. Moreover, collagen, specifically type I, enhances the adhesion and proliferation of cells, which is due to specific interactions with adhesion proteins^{6,7}. These findings could be attributed to the effect of increased initial cell attachment of PL-aAC as compared with PL, thereby increasing cell proliferation. Our results demonstrated that PL-aAC had an excellent initial response toward soft tissues as compared with PLGA. The latter crumbled in the initial stage, because it did not have sufficient mechanical strength to resist the pressure²⁷. In addition, PLGA degraded to acidic products and caused pH changes, which resulted in crumbling scaffolds and acidic products of PLGA lead to inflammation *in vivo*⁹.

Based on these results, the PL-aAC scaffolds may be suitable for the implant-type tissue-engineered bone. Also, the ideal absorption of biodegradable polymers should be synchronized with the bone regeneration. In future, we need further experiments to establish the ideal scaffolds by optimization of the biodegradability in the polymers. Additionally, we should accumulate the knowledge and experience of postoperative time-dependent changes for the 3D shapes of the tissue-engineered bone, not only in the immunocompromised animals, such as nude mice, but also in the rodents or the large-sized animals with normal immune systems.

CONCLUSION

The present results indicate that PL-aAC could be a better scaffold than PLGA for bone tissue engineering. Nevertheless, further *in vivo* evaluations are needed to test its ability to enhance new bone formation as identified in this preliminary investigation.

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