Fabrication of biodegradable \(\beta\)-tricalcium phosphate/poly(L-lactic acid) hybrids and their in vitro biocompatibility

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Hybrids of \(\beta\)-tricalcium phosphate (\(\beta\)-TCP) and poly(L-lactic acid) (PLLA) have been fabricated, which can be expected to be a novel filler for biodegradable bone grafting. Firstly, porous \(\beta\)-TCP ceramics have been made of calcium-phosphate fiber synthesized by homogeneous precipitation method. Then, the hybrids have been fabricated by introducing PLLA having high-molecular-weight into the open pores of the porous \(\beta\)-TCP ceramics. The mechanical property was enhanced, for example, by annealing the hybrids at 140°C for 24 h; the bending strength was 17.1 MPa, which was about 1.5 times that of the porous \(\beta\)-TCP ceramics. The biocompatibility has been examined using osteoblastic cell, MC3T3-E1, and found to be comparable to that of pure \(\beta\)-TCP ceramics.

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1. Introduction

In orthopedic surgery, bone grafting has been performed to treat diseases and injuries, such as bone tumor and bone fracture. In general, bone implantation is classed into three types: i) auto-grafting, ii) allo-grafting and iii) artificial-bone grafting. Among these, auto-grafting is well-known as a common practice in bone grafting, where an additional serious problem is involved that the donor bone should be supplied from healthy ilium or fibula of the patient by surgery.1,2 Alternatively, allo-grafting has been performed using donor bones obtained from bone banks; however, it also contains problems of supply, immunogenic factors and quality.2,3 Therefore, the artificial bone, which is integrated and/or replaced by the host’s newly-formed bone, is generally used for an artificial-bone grafting.3,4

As an artificial-bone graft, calcium phosphate ceramics, such as hydroxyapatite (Ca\(_{10}\)(PO\(_4\))\(_6\)(OH))\(_2\); HAp) and \(\beta\)-tricalcium phosphate (\(\beta\)-Ca\(_3\)(PO\(_4\))\(_2\); \(\beta\)-TCP), have been employed in orthopaedic surgery since the 1980ks.5 Numerous basic studies have demonstrated that HAp and \(\beta\)-TCP ceramics are biocompatible, bioactive and osteoconductive.6–11 However, the low solubility and slow in vivo resorption of HAp ceramics limit bone formation. On the other hand, \(\beta\)-TCP ceramics have some advantages when used as a bone graft, in that it is more rapidly resorbed.12

We have previously reported that calcium-phosphate fibers having long-axis sizes from 60 to 100\(\mu\)m can be prepared by a homogeneous precipitation method through heating the solution of Ca(NO\(_3\))\(_2\)-(NH\(_4\))\(_2\)PO\(_4\)-(NH\(_4\))\(_2\)HPO\(_4\)-(NH\(_4\))\(_2\)CO-HNO\(_3\)-H\(_2\)O.13–15 Subsequently, porous \(\beta\)-TCP ceramics with well-controlled open

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poles were fabricated by sintering these calcium-phosphate fibers.16–18 Most of the pores in the resulting porous ceramics were regarded as open pores, and the pore size could be controlled in the range of 0.2 to 5\(\mu\)m by varying the compaction pressure and firing temperature. Furthermore, larger pores of 20–150\(\mu\)m in diameter could be formed by using carbon beads as a porogen agent.16

If the mechanical property of the porous \(\beta\)-TCP ceramics can be matched closer to that of the human cortical bone, the clinical application of the porous \(\beta\)-TCP ceramics could be extended. Thus, in order to solve one of the problems, that is, the brittleness, we have tried to fabricate ceramics-polymer hybrids.

Since Bonfield et al.19 have produced a bone analogue hybrid of HAp particles in highly dense polyethylene (HAPEX™), which has been designed to provide bioactivity and to match the mechanical properties of human cortical bone, many ceramics–polymer hybrids have been developed by which excellent clinical performance has been achieved.20–27 For example, Kikuchi et al. have prepared HAp and collagen hybrids having a bone-like nanostructure, and reported excellent biocompatibility and bio-integrative activities.23,24 Sugiyama et al. have prepared the HAp/poly(L-lactic acid) (PLLA) hybrids by ring-opening bulk polymerization of L-lactide without additional catalysts, which have sufficient mechanical properties.25

In our group, Aizawa et al. have previously developed novel biodegradable hybrids consisting of \(\beta\)-TCP and PLLA, and reported good biocompatibility of the hybrids in vitro.26,27 The hybrids were fabricated by introducing PLLA into the porous \(\beta\)-TCP ceramics derived from calcium-phosphate fibers via ring-opening bulk polymerization of L-lactide monomer in the co-presence of lipase (enzyme polymerization). Mechanical properties of the resulting hybrids were similar to those of the human cortical bone. However, there was a problem that the
PLLA via this process had considerably lower molecular weight ($M_w$: 10,000–30,000) than the PLLA used for clinical application ($M_w$: 100,000–500,000).

In the present study, we have fabricated novel β-TCP/PLLA hybrids using commercially available PLLA with high-molecular-weight. The purpose of this study is to strengthen the porous β-TCP ceramics by keeping the highly-interconnected pore structures. In order to strengthen the porous β-TCP ceramics with small amounts of PLLA, we have annealed the resulting hybrids to increase crystallinity of PLLA. We will report some properties of the resulting hybrids, including in vitro evaluation using osteoblastic cell, MC3T3-E1.

2. Materials and methods

2.1 Fabrication of porous β-TCP ceramics and their characterization

Calcium-phosphate fibers as a starting material were synthesized by a homogenous precipitation method from the solution of Ca(NO$_3$)$_2$·(NH$_4$)$_2$HPO$_4$·(NH$_4$)$_2$CO·HNO$_3$·H$_2$O. The starting aqueous solution with a Ca/P ratio of 1.67 was prepared by mixing Ca(NO$_3$)$_2$, (NH$_4$)$_2$HPO$_4$, (NH$_4$)$_2$CO and HNO$_3$ so that their concentrations were 0.167, 0.100, 0.500 and 0.10 mol·dm$^{-3}$, respectively. The starting solution (0.75 dm$^3$) was refluxed at 80 °C for 48 h to synthesize calcium-phosphate fibers having long-axis sizes from 60 to 100 μm. The fiber fractions were easily separated by decantation; the yield of the fibers was in range of 50–60%.

Porous β-TCP ceramics were fabricated as follows. Firstly, the resulting calcium-phosphate fibers were put into a stainless steel mold and uniaxially compressed at 30 MPa to form compacts. The dimensions of the compacts were 6.0 mm × 40.0 mm × 2.0 mm for three-point bending test, and 15.0 mm in diameter and 2.0 mm in thickness for in vitro evaluation. Then, porous β-TCP ceramics were fabricated by firing the compacts at 1000°C for 3 h in air atmosphere; the heating rate was 10°C·min$^{-1}$.17,18

The crystalline phases of the resulting ceramics were identified by X-ray diffractometry (XRD: Rigaku, MiniFlex, 30 kV, 15 mA, Cu Kα$\gamma$ radiation). The functional groups were detected by Fourier-transform infrared spectroscopy (FT-IR; Shimadzu, IR Prestige-21, measurement range: 400–4000 cm$^{-1}$).

2.2 Fabrication of β-TCP/PLLA hybrids and their characterization

Hybrids of β-TCP and PLLA were fabricated by introducing 2 mass% PLLA solution into the open pores of the porous β-TCP ceramics. Commercially available PLLA with high-molecular-weight (Boelinger Ingelheim, Resomer® L 210 S, $M_w$: 300,000) was dissolved in chloroform under continuous stirring to attain PLLA (chloroform) solution of 2 mass%; the concentration is here expressed in the mass ratio of solute/solvent. The porous β-TCP ceramics (about 0.4–1.0 g) were immersed into the 2 mass% PLLA solution (about 20–30 cm$^3$); then the remaining air inside the pores was replaced as much as possible by the PLLA solution with ultrasonic treatment (38 kHz) for 10 min. The effect of repeating the procedure (the insertion of PLLA into the ceramics + drying) was examined totally 3 times or 5 times for some groups of the specimens. The porous β-TCP ceramics filled with the PLLA solution were dried at room temperature for 1 week to remove the residual solvent.

Some parts of the resulting β-TCP/PLLA hybrids were annealed in an oven at 140°C for 24 h. Other parts of the specimens were heated in the oven at 200°C for 30 min to melt the PLLA, and then annealed at 140°C for 24 h. The β-TCP/PLLA hybrids without the annealing process will be named “hyb-0(0)”. Annealed β-TCP/PLLA hybrids after the melting process will hereafter be named “hyb-n(+)+”, and annealed β-TCP/PLLA hybrids without melting process will be named “hyb-n(−) (+) (n: introduction times of PLLA). These abbreviations of β-TCP/PLLA hybrids are listed in Table 1.

The intended aim of the annealing process was to increase crystallinity of PLLA and to enhance the mechanical property of the β-TCP/PLLA hybrids.28 By melting PLLA before the annealing process, stronger bonding between PLLA and porous β-TCP ceramics was expected.27

The three-point bending strength of the resulting β-TCP/PLLA hybrids was measured using a 50N load cell at a crosshead speed of 0.5 mm·min$^{-1}$ (Shimadzu, Autograph AGS-J). The tested specimens were in the rectangular shape of approximately 6.0 mm × 40.0 mm × 2.0 mm. Young’s modulus was calculated from the slope of the load-distortion curve of the three-point bending test. Three specimens were measured for the average value and the standard deviation (S.D.).

The relative density ($d_{rel}$) of the β-TCP/PLLA hybrids is defined by

$$d_{rel} = 100\left(\rho_{bulk}/\rho_{true}\right)$$

where $\rho_{bulk}$ and $\rho_{true}$ are the bulk density and the true density of the specimens, respectively. The total porosity ($P_{total}$) of the β-TCP/PLLA hybrids was evaluated by

$$P_{total} = 100 – d_{rel}$$

The $\rho_{true}$ was measured at 31.0 ± 0.2°C using a pycnometer, where ethanol was used as the immersion liquid.

The microstructures of the β-TCP/PLLA hybrids were observed by scanning electron microscopy (SEM; JEOL, JSM-6390LA) at an accelerating voltage of 5 kV. The specimens were coated with platinum by an auto fine coater (JEOL, JFC-1600) before the observation.

2.3 Crystallinity of the annealed high-molecular-weight PLLA

The films of high-molecular-weight PLLA were prepared by casting 2 mass% PLLA (chloroform) solution. The polymer solution was poured into a glass-dish and the solvent was evaporated slowly in the air at room temperature for 1 week to prevent the formation of air bubbles. As-prepared PLLA films (PLLA(0)) were annealed in the oven at 140°C for 24 h (PLLA(−)). Some parts of the specimens were melted at 200°C for 30 min, and annealed at 140°C for 24 h (PLLA(+)). The crystallinity of these PLLA films was determined by XRD. Number-average molecular weights ($M_n$) of these PLLA films were determined by gel permeation chromatography at 40°C, with chloroform as the mobile phase. Polystyrene standards were used to calibrate the results.
2.4 In vitro evaluation using the osteoblast model
2.4.1 Cell culture
Osteoblast-like MC3T3-E1 cells were used for in vitro evaluation of the β-TCP/PLLA hybrids. The MC3T3-E1 cells were cultured in alpha modified Eagle’s minimum essential medium (α-MEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO) (α-MEM(+), hereafter) in 25 cm² flasks (Nunc). The culture medium was changed every two days. The cells were maintained at 37 °C in a fully humidified atmosphere containing about 5% CO₂ in volume. When nearly-confluent, the cells were passaged with 0.1% actinase (Kaken pharmaceutical) dissolved in Dulbecco’s phosphate-buffered saline solution without calcium or magnesium salts (PBS(−), pH 7.4), and counted with a hemocytometer. Cell suspensions were prepared in the above-mentioned culture medium and adjusted to give 5.0 × 10⁴ cells cm⁻³. The cell suspensions of 1 cm³ were seeded on three kinds of specimens: i) porous β-TCP ceramics, ii) hyb-3(0) and iii) hyb-3(+), after soaking these specimens in the above-mentioned medium for 24 h. A polystyrene tissue culture plate having 24 wells (IWAKI) was used as a control in order to assay the cell viability of MC3T3-E1 cells cultured in the present system. Disk-shaped specimens (Ø15.0 mm × 2.0 mm) were used for the in vitro evaluation, and the disks were polished with #2000 SiC paper and cleaned with an ultrasonic cleaner before use. Therefore, the culture surface of the β-TCP/PLLA hybrids is composed of both β-TCP and PLLA. All the specimens used for the in vitro evaluation were sterilized with ethylene oxide gas (EOG 20%; CO₂ 80% in weight) (Iiken, SEMMEL 502B).

2.4.2 Initial cell-attachment and cellular proliferation
The initial cell-attachment of MC3T3-E1 cells was determined using the hemocytometer. After 5 h of culture, the cells were washed three times with PBS(−) and harvested by a 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) solution (GIBCO) and following pipetting. The trypsin/EDTA solution was neutralized with an equal volume of α-MEM(+), followed by 5 min of centrifugation at 1000 rpm, and then the cell pellets were suspended in PBS(−) and the number of the cells were counted using the hemocytometer. Initial cell-attachment (Ainit) of MC3T3-E1 cells is defined by

\[ A_{\text{init}} \% = 100(N_2/N_0) \]  

where \( N_2 \) and \( N_0 \) are the numbers of cells 5 h after seeding and at seeding on the specimens, respectively.

The proliferation of MC3T3-E1 cells was also determined using the hemocytometer. After 1, 3, 5 and 7 days of culture, the number of cells was counted by the method described in Section 2.4.2. The culture medium was changed every two days during the evaluation. Doubling time (\( T_d \)) of the cell during the logarithmic growth phase (1–3 d) is defined by

\[ T_d \ (h) = (48 \ h) \log_{10} 2/(\log_{10} N_3 - \log_{10} N_1) \]  

where \( N_3 \) and \( N_1 \) are the numbers of cells 3 days and 1 day after seeding on the specimens, respectively.

2.4.3 Cell morphology
Cell morphology was observed by SEM at 1, 3, 5 and 7 days of culture for three specimens. Cell morphology of the control was observed by a phase-contrast microscope. After 1, 3, 5 and 7 days of culture, cells were washed three times with PBS(−) and fixed with 10% glutaraldehyde in PBS(−) at 4 °C for 1 h. Then, the glutaraldehyde solution was removed and the cells were washed with PBS(−) and subsequently with distilled water three times.

Fig. 1. (A) XRD pattern and (B) FT-IR spectrum of the porous β-TCP ceramics.

The cells were then freeze-dried and coated with platinum by an auto fine coater. The specimens were examined under SEM at an accelerating voltage of 5 kV.

2.4.4 Statistical analysis
Values of cell activity were expressed as the average ± S.D. of at least three specimens. When the results are compared among the groups, these were considered to be statistically different when \( P < 0.02 \) using the one-way analysis of variance (ANOVA).

3. Results and discussion
3.1 Characterization of β-TCP/PLLA hybrids
Figure 1 shows the XRD pattern (Fig. 1(a)) and the FT-IR spectrum (Fig. 1(b)) of the porous ceramics, whose fabrication has been described in Section 2.1. The powdered porous ceramics were used for the XRD and the FT-IR measurements. The XRD pattern reveals that a single phase of β-TCP was present in the sample powder. The FT-IR spectrum showed absorptions at 1300–900, 600 and 550 cm⁻¹, assignable to the PO₄³⁻ group of β-TCP. By-products expected from decomposition of calcium-phosphate fibers, such as HA, were not detected. The above results show that the porous ceramics fabricated from calcium-phosphate fibers were composed of the β-TCP single phase.

Figure 2 shows the total porosity of the resulting β-TCP/PLLA hybrids (hyb-1(0), hyb-3(0), and hyb-5(0)), together with that of the porous β-TCP ceramics. Total porosities of the porous β-TCP ceramics were 53.8 ± 0.5%. In contrast, after introducing
PLLAs into the porous β-TCP ceramics for 1 time and 3 times (hyb-1(0) and hyb-3(0)), total porosities of the porous β-TCP ceramics slightly decreased to 52.1 ± 0.6% and 51.0 ± 0.8%, respectively. When PLLA was introduced into the porous β-TCP ceramics, no significant difference in porosities was detected between hyb-3(0) and hyb-5(0), as for the abbreviations, see Table 1. These results indicate that hyb-3(0) has reached the maximum amount of PLLA that could be introduced into the porous β-TCP ceramics by the present process (amount of PLLA introduced into hyb-3(0) was 2.54 mass %). Therefore, the β-TCP/PLLA hybrids (hyb-3(0), hyb-3(−) and hyb-3(+)) were used for further study.

Figure 3 shows the SEM images of (a) calcium-phosphate fibers and (b) porous β-TCP ceramics. It can be seen from Fig. 3(a) that the calcium-phosphate fibers have long-axis from 60 to 100 µm. Highly-interconnected pore structures of the porous β-TCP ceramics, which originate from fiber-shape of the calcium-phosphate fibers could be observed from Fig. 3(b). Pore sizes ranged from 1 to 5 µm.

Figure 4 shows the microstructures of (a) hyb-3(0), (b) hyb-3(−) and (c) hyb-3(+). Figures 4(a'), (b') and (c') are the more magnified images in Figs. 4(a), (b) and (c), respectively. The arrows indicate the fragments of the PLLA films formed on the pore surfaces of the β-TCP/PLLA hybrids. Figures 4(a)-(a') reveal that the pore surfaces of the β-TCP ceramics have become rougher by the coated layer of PLLA. The fragments of the PLLA films were partially observed on the pore surface of hyb-3(0). Figures 4(b)-(b') show that no appreciable difference in the surface structures is observed after the annealing process. On the other hand, Figs. 4(c)-(c') demonstrate that the surface structure of the annealed hybrids after the melting process (hyb-3(+)) were smoother than that of hyb-3(0) and hyb-3(−). In addition, no fragments of PLLA are observed. The highly-interconnected pore structure of the porous β-TCP ceramics is maintained after the melting or the annealing processes.

By the melting process, coated layer and fragments of PLLA on the pore surface have melted once, and then the coated layers of PLLA become more uniform. As a result, the bindings between PLLA and porous β-TCP ceramics have become stronger. If the coating layer is non-uniform, the parts may become the origin of fracture, which will, in turn, lead to a decrease in the mechanical strength. Therefore, the surface structure of hyb-3(+) is supposed to be preferable to the present purpose.

3.2 Crystallinity of the annealed high-molecular-weight PLLA

Figure 5 shows the X-ray diffraction patterns of PLLA films before and after the annealing process. In Fig. 5(a), the PLLA(0) has a broad diffraction pattern which is specific to amorphous polymeric materials. Crystalline peaks are observed at 2θ = 17.8° for the PLLA(−) and PLLA(+), as shown in Figs. 5(b) and (c). The crystalline peak of PLLA(+) is much stronger than that
of PLLA(−). Tsuji and Ikeda found that, if PLLA having high molecular weight is melted, nucleation proceeds upon quenching and that, therefore, higher nucleation density is achieved. In the present case of Fig. 5(b) without melting (PLLA(−)), nucleation occurs to some extent with the annealing process. Figure 5(c) shows, however, the effect of melting on nucleation is much greater than that of annealing. These results indicate that the crystallinity of PLLA could be also controlled by skipping the melting process or not.

The $M_n$ of the PLLA films were 392,000 for PLLA(0), 375,000 for PLLA(+) and 264,000 for PLLA(−). $M_n$ of PLLA(0) decreased to 96% by the annealing process (PLLA(−)), and to 67% by the annealing process after the melting process (PLLA(+)). Generally, mechanical strength of the polymer materials increases as the $M_n$ gets higher, and will reach the plateau in due course. Consequently, mechanical strength of the polymer with high-molecular-weight would hardly depend on $M_n$. As the $M_n$ more than 200,000 were maintained still after the melting process and annealing process (PLLA(+)), it could be said that the decrease in $M_n$ caused by the present heating process is less effective to the mechanical strength of PLLA. Therefore, crystallinity of PLLA strongly gives an effect to mechanical strength of the hybrids than molecular weight.

### 3.3 Mechanical properties of $\beta$-TCP/PLLA hybrids

Figure 6 shows (A) three-point bending strength and (B) Young’s modulus of (a) porous $\beta$-TCP ceramics, (b) hyb-3(0), (c) hyb-3(−) and (d) hyb-3(+). No significant difference was recognized in the bending strength of (a) and (b). On the other hand, the bending strength of (c) and (d) was significantly higher than that of (a), while no significant difference was detected between the two annealed $\beta$-TCP/PLLA hybrids ((c) and (d)). Young’s modulus of the specimens was in the following order: porous $\beta$-TCP ceramics, hyb-3(+) > hyb-3(−) > hyb-3(0). Young’s modulus of the $\beta$-TCP/PLLA hybrids correspond to the crystallinity of PLLA, which is shown in Fig. 5.

As mentioned above, the amount of PLLA introduced into hyb-3(0) was 2.54 mass %, which led to the decrease of 2.8% in the porosity. Therefore, the amount of PLLA introduced into the porous $\beta$-TCP ceramics was too low to produce an effect on the three-point bending strength of the porous $\beta$-TCP ceramics. By annealing the $\beta$-TCP/PLLA hybrids, crystallinity and the stiffness of the introduced PLLA have been improved, which has brought higher three-point bending strength to the hybrids.

### 3.4 Biocompatibility of the $\beta$-TCP/PLLA hybrids using MC3T3-E1 cells

For the cytotoxicity test, we examined initial cell-attachment, proliferation and cell morphology of MC3T3-E1 cells seeded on four kinds of specimens: (a) control (24-well polystyrene tissue culture plates), (b) porous $\beta$-TCP ceramics, (c) hyb-3(0) and (d) hyb-3(+). In order to determine the difference of biocompatibility caused by the crystallinity of PLLA, hyb-3(0) and hyb-3(+) (cf. Table 1) were chosen for the in vitro examination. Figure 7 shows the initial cell-attachment efficiency of the MC3T3-E1 cells cultured on four kinds of above-mentioned specimens. No significant difference was recognized among the $\beta$-TCP/PLLA hybrids by one-way ANOVA ($P < 0.02$). However, initial cell-attachment efficiency of porous $\beta$-TCP ceramics indicated lower than that of the $\beta$-TCP/PLLA hybrids. This may be because the surface wettability of the porous $\beta$-TCP ceramics changed from hydrophilic to hydrophobic through the introduction of PLLA, which made it easier for the cells to attach to the
Figure 7 shows the cell morphologies of the MC3T3-E1 cells cultured for 5 days on the four kinds of specimens referred to in Fig. 8. Figure 9(a) shows the phase-contrast microscope image of the MC3T3-E1 cells cultured on a tissue culture plate, where the cells were nearly confluent. Figures 9(b), (c) and (d) show the SEM image of the MC3T3-E1 cells cultured on the porous β-TCP ceramics, hyb-3(0) and hyb-3(+), respectively. The MC3T3-E1 cells cultured on all the specimens have well-proliferated and fully-stretched over the porous structure of the specimens. No difference in cell morphology among the specimens was observed.

The above-mentioned results for in vitro evaluation using MC3T3-E1 cells demonstrate that the present β-TCP/PLLA hybrids as well as the porous β-TCP ceramics had no cytotoxicity. The present β-TCP/PLLA hybrids may be applied as novel fillers for biodegradable bone grafting.

4. Conclusions

We have successfully fabricated β-TCP/PLLA hybrids from biodegradable materials: β-TCP and PLLA, and examined the mechanical property and the biocompatibility of the resulting hybrids. The bending strength of the annealed β-TCP/PLLA hybrids has attained about 1.5 times that of the porous β-TCP ceramics. Examination of the cell growth has shown that the MC3T3-E1 cells cultured on the porous β-TCP ceramics and β-TCP/PLLA hybrids have good proliferation. We conclude that the present β-TCP/PLLA hybrids may be a high-performance artificial bone graft.

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