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Evaluation of Bone Regeneration of Apatite Coating Poly-L-lactide Scaffold in Rat Calvarial Defects

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Synopsis
This study examined in vivo biocompatibility of poly-L-lactide (PLLA) scaffolds coated with hydroxyapatite (HA) by pulsed laser deposition (PLD). A thin HA film was made on PLLA discs by an ArF PLD operating at a repetition rate of 10 Hz. Cranial bone defects were created in rats and filled with non-HA-coated PLLA scaffold (PLLA) or HA-coated PLLA scaffold (HAPLLA). All specimens were evaluated micro-radiographically and with histological analysis at 2, 4, and 6 weeks. The presence of calcium and phosphorous on the surface as well as in the interior of the PLLA was confirmed by energy dispersive analysis. In vitro cell-spread tests showed that the human osteoblasts spread more on HAPLLA than on PLLA. Upon transplanting calvarial defects, the in vivo hard tissue responses suggested earlier restoration of the defects with HAPLLA than with PLLA.

Key words: bone tissue engineering, hydroxyapatite, poly-L-lactic acid, pulsed laser deposition, rat calvarial defect

Introduction
Cleft lip and palate is a frequently occurring congenital malformation that is caused by genetic and environmental factors [1, 2]. Loss of alveolar bone due to the cleft can lead to problems with feeding and speech, among other difficulties; therefore, surgical closure is strongly recommended [3, 4]. Autogenous bone grafting for patients with cleft lip and palate has become a well-accepted treatment modality to restore the function and structure of the maxillary arch at the cleft site [5, 6]. However, the procedure is very invasive and the amount of collectable bone is limited. Allogeneic bone grafts may transmit diseases and can cause immune-related complications. It is therefore necessary to develop a synthetic alternative to current graft materials for bone regeneration [7].

In recent years, poly(L-lactide) (PLLA) has been widely evaluated as a scaffold biomaterial because of its impressive biocompatibility, biodegradability, minimal inflammatory reaction, and excellent mechanical properties [8]. However, PLLA is known to show poor cell–material interaction because of its hydrophobic nature and lack of cell recognition signals [9]. In order to promote cell adhesion, surface modification of PLLA is often attempted [9].
As a major component of the extracellular matrix of bone, hydroxyapatite (HA) is widely used in orthopedic and maxillofacial surgery, because it has high biocompatibility and osteo-compatibility [10]. Coatings of bioactiveapatite, which is similar to bone apatite, have therefore often been used in bone tissue engineering [11]. In the present study, HA coatings were manufactured by pulsed-laser deposition (PLD). PLD methods allow the deposition of a very thin coating, as well as control of surface roughness and ablation of any material, thus providing strong bonding between the coating and substrate [11-13].

The PLLA mesh scaffold used in this study, Biofelt™ (Corefront, Tokyo, Japan), is a complex three-dimensional (3D) material. It was thought that it might be difficult to deposit an HA layer throughout the inner region of the scaffold. In our previous study, HA was coated onto a porous titanium wave (TW) scaffold, comprising intertwined titanium fibers, using the PLD technique. The homogeneity of the extent of bone growth in pores inside the implant was investigated, and it was reported that the thin apatite coating was deposited not only on the surface, but also within the TW inner region [11]. To our knowledge, this is the first time the PLD technique has been used in coating the PLLA mesh scaffold, which is frequently used in tissue engineering.

In this study, we fabricated an HA-coated PLLA (HAPLLA) scaffold for the regeneration of alveolar bone defects by the PLD technique. The coating quality and in vitro biocompatibility were investigated. The effects of HAPLLA on bone regeneration in surgically created rat calvarial defects were then evaluated using micro-computerized tomography (CT) and histological examination.

Materials and methods

1. Preparation of experimental materials

BIOFELT (PLLA) used in this study was purchased from corefront™. HA (Japan Chemical Co., Sapporo, Japan) powder was pressed at 150 MPa in a cylindrical steel die to form disks with thickness and diameter of 5 mm and 2 mm, respectively. These HA disks were used as targets for PLD. The HA target was placed in the vacuum chamber. Molecules, atoms, and ions were desorbed from the target surface in a vacuum chamber by irradiation with an ArF excimer laser (Lambda Physik LPX-210icc; λ = 193 nm; full width at half maximum of pulse = 20 ns). This phenomenon is called laser ablation. The laser fluence was about 1 J / cm² and the pulse repetition rate was 10 Hz. The deposition rate was about 10 nm / min at room temperature. During the deposition, O₂ gas bubbling through a pure water-bath was introduced into the vacuum chamber with a base pressure of about 10⁻⁴ Pa. Water molecules were introduced into the chamber by the bubbling; thus, the OH group was supplied for HA. The desorbed molecules, atoms, and ions react with the atmosphere gas (O₂ + H₂O; partial pressure approximately 0.1 Pa) at the substrate. HA was coated from 6 directions: at normal directions to both surfaces and at ±45º to normal for both surfaces. Coating from 6 directions ensures a more homogeneous thickness even deeper within the PLLA. The typical average deposition rate was 0.8 nm / min. Because the deposition time for each direction was 40 min, the total deposition time was 4 h. The microstructures of the obtained grafting materials were then observed using a scanning electron microscope (SEM) (S-450; Hitachi Ltd, Tokyo, Japan).

2. SEM observation of HAPLLA and PLLA

The morphology of the HAPLLA was examined using an SEM (S-4800). Samples were dehydrated in ascending grades of ethanol, dried, and mounted on an aluminum stub using double-sided carbon tape. The specimens were coated with vanadium using a sputter coater and examined with SEM at an accelerating voltage of 10 kV.

3. EDX analysis of the surface area and inner region of HAPLLA

The effectiveness of the HA coating on the surface or inside the PLLA was evaluated by energy dispersive x-ray analyzer (EDX; KRA8800, Ametek Co., Tokyo, Japan) at an accelerating voltage of 25 kV by detecting the x-ray intensities of Ca-Kα, P-Kα, and Ti-Kα. In this study, the EDX results are corrected with the standardless ZAF (Z, atomic number; A, absorption co-
efficient; F, fluorescent excitation effect) method. This correction shows the deviation of the measured chemical composition from the true value of the sample.

4. Morphology of MG63 cells
The MG63 cell line was originally derived from a human osteosarcoma and has been shown to exhibit many characteristics of pre-mature osteoblasts, making it an attractive model for in vitro studies [14-16]. The cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and maintained by continuous culture at 37 °C in a 5% CO₂ humidified atmosphere. MG63 cells were expanded for 3–5 days in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated serum and 1% antibiotic / antimycotic in 75 cm² flasks. When monolayers reached confluence, the cells were enzymatically lifted from the flask with trypsin. MG63 cells (n = 20,000) were seeded onto bare PLLA (non-HA film) or onto PLLA deposited with a thin coating of HA (HA film) in 96-well tissue culture plates containing DMEM supplemented with 10% fetal bovine serum. The cells were incubated for 24 h in a CO₂ incubator at 37°C and fixed using 4% paraformaldehyde solution for 20 min at room temperature. Following fixation, the cells were permeabilized using 0.2% Triton-X-100 for 2 min and incubated for 30 min with Alexa Fluor rhodamine–phalloidin solution, which binds specifically to actin filaments. They were then washed 5 times with phosphate-buffered saline and analyzed by automated fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).

5. Surgical procedure
Male Sprague–Dawley rats (n = 27, age 12 weeks, weight 350–370 g) were used for transplantation. The rats were anesthetized with 30 mg / kg intraperitoneal injection of pentobarbital sodium (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan) in addition, 0.2 mL of local anesthetic (1% xylocaine/epinephrine 1:100,000; Astra-Zeneca, Tokyo, Japan) was injected into the surgical sites before the start of surgery. The surgical areas were shaved and disinfected with povidone iodine (Isodine Surgical Scrub; Meiji, Tokyo, Japan). Skin incision and subperiosteal dissection were then carried out. After the flap was raised, a critical-sized bone defect (diameter, 5 mm; depth, 1.0 mm) was created at the center of the skull using a bone trephine bur (external diameter, 5 mm) under running water. The rats were divided into 3 groups for the experiments: in one group, the defect was filled with HAPLLA; on another, the defect was filled with PLL only; and in the control group, the defect was not filled with any material. In all groups, the defects were covered using a GORE-TEX® GTR membrane (Japan Gore-Tex Co., Tokyo, Japan). Finally, the flap was repositioned and sutured. The skin was sutured with 3-0 silk (Natsume Seisakusho Co., Tokyo, Japan). The animals were euthanized at 2, 4, and 6 weeks after surgery (n = 3 at each time interval per group).

6. Micro-CT and three-dimensional color mapping
Rats were euthanized 2, 4, or 6 weeks after scaffold implantation. Calvarial bone was excised, trimmed, and fixed in 4% paraformaldehyde for 24 h at 48 °C. The specimens were examined using a micro-CT machine (Shimadzu, Kyoto, Japan). Blocks of bone specimens were mounted on a turntable by using utility wax. The exposure conditions were 65 kV and 90 μA at 24.7-μm intervals for 21.1-μm-thick slices. The information from all the slices was saved at 512 × 512 pixels. The linearity of the micro-CT scanner was established by scanning a phantom containing several densities of a standard calibration material. Three-dimensional (3D) measurements of calvarial bone were obtained, and its structural indices were calculated using a morphometric program (TRI/3D-BON; Ratoc System Engineering, Tokyo, Japan). For bone-density measurements, an HA calibration curve was prepared from the images of phantoms (HA content: 200, 300, 400, 500, 600, 700, and 800 mg / cm³).

7. Bone volume / total volume, bone mineral content, and bone mineral density analysis
New bone was measured using the TRI/3D-BON 3D analysis routine using the obtained CT values. In the 3D analysis, the total volume (TV, cm³), bone volume (BV, cm³), and bone mineral content (BMC, mg) were measured directly. The volumetric density was calculated as BV/TV (%),
and the bone mineral density (BMD) was calculated as BMC/BV (mg/cm³); these indices evaluated the quantity and density of new bone, respectively.

8. **Histological analysis (hematoxylin and eosin staining)**

   Histological examination was carried out to observe the tissue structures and thicknesses of the new bone. Samples were fixed with 10% phosphoric acid in neutral buffered formalin for 2 weeks. They were processed subsequently for histological examination according to standard procedures—the samples were decalcified, embedded in paraffin, sectioned at 4 mm thickness, and subjected to hematoxylin and eosin (HE) staining. Each section was examined using a BZ9000 All-in-One Fluorescence Microscope (BZ-9000; Keyence Co., Osaka, Japan).

9. **Statistical analysis**

   All data were expressed as the mean and standard error. Differences were evaluated by analysis of variance (ANOVA) with Scheffe’s F test. Differences were considered significant at p < 0.05.

**Results**

1. **SEM observation of HAPLLA and PLLA**

   The microstructures of PLLA and HAPLLA are shown in Fig. 1. The 3D porous structures of both HAPLLA and PLLA were clear, and their structures were very similar. HA could be seen adhered to and extending over the PLLA surface.

2. **EDX analysis of the surface area and inner region of HAPLLA**

   The presence of HA coating not only on the sur-

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**Fig. 1** Scanning electron micrographs of PLLA (A, B) and HAPLLA (C, D).

Original magnification: ×50; original magnification: ×500
face, but also on the inner region of the PLLA was confirmed by the EDX curve of calcium and phosphorus (Fig. 2). Within the PLLA, HA coating was uniform; however, Ca and P concentrations were lower than those at the surface (Fig. 2).

3. Morphology of MG63 cells
The ability of MG63 cells to organize the actin cytoskeleton during culture was investigated. Microscopic images after actin staining with phalloidin showed that there were more MG63 cells on untreated PLLA fibers than on untreated PLLA fibers after 24-h incubation (Fig. 3). The number of MG63 cells was greater on HA-coated surfaces than on untreated surfaces (Fig. 3).

4. Micro-CT and 3D color mapping
Fig. 4 shows a representative BMD distribution map of the 3D map and micro-CT images of bone regeneration. Each model was analyzed at 3 time points: 2, 4, and 6 weeks after surgery. The 3D map shows the BMD at these time points. The BMD color scale is as follows: red and orange = high BMD; yellow and green = medium BMD; and light blue and purple = low BMD.

Results, 2 weeks after surgery: The control groups had a low BMD value (blue color). Micro-CT images showed that the defects were not filled with hard tissue and the bone defect remained. In contrast, a low-to-medium BMD value (green color) was observed in both the HAPLLA and PLLA groups. Micro-CT images showed that defects were filled with small amounts of hard tissues (Fig. 4A).

Results, 4 weeks after surgery: The control and PLLA groups had a moderate BMD value (blue and green color). In the control group and PLLA groups, micro-CT images showed that defects were not completely filled with hard tissue. In the PLLA group, more hard tissue was observed than in the control group. In contrast, a medium BMD value (yellow color) was observed in the HAPLLA group (Fig. 4B).

Results, 6 weeks after surgery: The control and PLLA groups had a moderately high BMD value (purple and green color). Micro-CT images showed that defects were filled with hard tissue, and the amount of hard tissue in this group had clearly increased with time (Fig. 4C).

5. BV/TV, BMC, and BMD analysis
The BV/TV for each group at each time is shown in Fig. 5A. At 2 weeks post-transplant, there was a significant difference between BV/TV in the HAPLLA groups and the PLLA
Fig. 3 Microscopic images after actin staining with rhodamine–phalloidin of cells cultured for 24 h on PLLA before or after HA coating. Scale bars: 100 mm.

Fig. 4 Micro-CT images and the BMD distribution map of the 3D map at 2, 4, and 6 weeks after surgery.

Fig. 6 Histological micrographs of HE-stained sections from each group at 2, 4, and 6 weeks after surgery. Original magnification: ×200. Scale bars: 100 mm.
group. At 4 weeks post-transplant, BV/TV in the HAPLLA group was greater than the values in the other groups. At 6 weeks post-transplant, BV/TV in the HAPLLA group was the highest among the 3 groups. At 6 weeks HAPLLA, there were significant differences in the BT/TV among the PLLA and control groups.

The new bone BMD and BMC of each group is shown in Fig. 5 (B, C). This reflects new bone density and mineralization at each time point. At 4 weeks, BMC of the HAPLLA group was higher than that of control group. At 6 weeks, there were significant differences in the BMD and BMC between the HAPLLA group and the PLLA and control groups.

6. Histological analysis (HE staining)

Results 2 weeks after surgery: In both PLLA and HAPLLA groups, acute inflammatory and necrosis responses were not observed in the connective tissues surrounding the scaffold. In the PLLA group, the PLLA was not in contact with the original bone. In the HAPLLA group, HAPLLA was absorbed, surrounded by connective tissue and capillaries from the original bone, and a small amount of new bone tissue was formed from the original bone (Fig. 6A, B).

Results, 4 weeks after surgery: In the both PLLA and HAPLLA groups, the bone defects were filled with dense connective tissue and the remaining PLLA. A small fraction of osteoid was apparent on the original bone site. In addition, small island-shaped bone was clearly formed in graft areas (Fig. 6C, D). In the HAPLLA group, the proliferated connective tissues contained more immature bone tissue, including osteocytes, than in the PLLA group. The new bone was formed on the original bone side of the skull (Fig. 6D).

Results, 6 weeks after surgery: At this time point, the remaining PLLA fibers were still clearly observed in the both groups. However, considerable absorption of PLLA fibers in contact with the original bone was observed in the HAPLLA group (Fig. 6E, F). The new bone was more frequently observed on the pericranium side, and many osteoblasts were observed around the new bone (Fig. 6F).

![Graphs](image-url)
Discussion

The present study showed that the PLD method is effective in depositing an HA coating within porous scaffold materials such as Biofelt™, as well as on the surface. HA coating of PLLA also enhanced cell attachment and proliferation. The in vivo study demonstrated that apatite-coated PLLA did not cause any adverse responses and showed earlier restoration upon transplanting for calvarial defects. However, micro-CT examination did not show notable bone formation in the treatment of bone defects.

Many porous scaffolds are composed of biodegradable polymers and bioactive ceramics. HAPLLA composite has become an important porous scaffold, since it combines the osteoconductivity and bone-bonding ability of HA with the resorbability and the processing ease of the PLLA [17, 18]. In order to bond the HA onto the PLLA fabric, atoms and ions desorbed from the HA target surface in a vacuum chamber were introduced on the PLLA surface by ArF excimer laser. Vehof et al. [19] deposited a thin calcium phosphate coating onto a porous titanium scaffold using an RF magnetron sputtering technique. The RF magnetron sputter coating did not completely penetrate the fiber structure of the scaffold, and the insufficient deposition in the inner region was concluded to have influenced the bone response. Vehof et al. [19] used a method of coating employing droplet spraying: a homogeneous 3D infusion could not be achieved because of clogging at the surface fibers. The PLD technique, which deposits the HA film in the form of atomic or molecular size species, might overcome this clogging problem [11]. Ablated chemical species collide with the ambient O2/H2O molecules, inducing them to diffuse to the back side of the Ti fibers and encouraging homogeneous coating of the HA onto the PLLA scaffold [11]. EDX analysis indicated that the HA had been coated homogeneously, even deep within the porous structure. In addition, the plasma spray technique involves HA being melted at a high temperature, which cannot be tolerated by PLLA.

The actin cytoskeleton of cells on the HA surfaces was well organized. Thus, the organization of the actin cytoskeleton was positively influenced by the HA surfaces. The surface characteristics of HA improved short- and long-term responses, such as initial attachment and the differentiation of bone marrow cells in vitro. These behaviors could be explained by the adsorption of serum fibronectin and vitronectin [20]. The adhesion morphology and the degree of cell spreading depends on the kinds of previously adsorbed proteins on the biomaterials [20]. Thus, fibronectin and vitronectin were able to bind to cell integrins and enhance cell attachment on biomaterials in vitro. It is also suggested that HAPLLA can enhance cell attachment and proliferation because of adsorption of proteins such as fibronectin and vitronectin onto the HAPLLA. In the present study, the HAPLLA made through the PLD technique might have low crystallinity, since the samples could not be heated to cause annealing. This is in agreement with previous reports that the low crystallinity of the HA coating could stimulate the proliferation of cells [21].

In this study, the osteoconductivity and biodegradation properties of a PLLA material were evaluated using a rat calvarial defect model. The calvarial bone consisted mainly of cortical bone with minimal amounts of bone marrow cells. This provided a limited source of cells of osteogenic lineage compared with other models that use different bone sites such as the femur, where periosteal, endosteal, or bone stromal cells and their interactions are involved in healing [22]. This experimental model was therefore suitable to test the osteoconductivity of the implant material in a simulated model of bone regeneration of an alveolar cleft defect.

Micro-CT has a high resolution and uses very thin slices. A larger number of slices means that the spatial differences within areas of the specimen become negligible [23]. The geometrical properties of the healing bone can be acquired, along with the spatial distribution of BMD, by simultaneous scans of a calibration phantom using micro-CT [23]. The BMD distribution map may be useful for evaluating the regeneration of new bone. We selected the BV/TV, BMD, and BMC of mineralized tissue, comprising the bone quality, as the denominator along with the bone volume. In our study, micro-CT analysis showed that BV/TV, BMD, and BMC in...
the HAPLLA group were the highest among the 3 groups at 6 weeks post-transplant. The bone quantity and quality of the experimental group had increased considerably compared with those of the control and PLLA groups. These results suggested that HAPLLA promoted new bone formation. However, the value of BV/TV in the HAPLLA group was less than 50%, and notable bone formation was not occurred.

Newly formed small bone trabeculae were observed in the proliferated connective tissue adjacent to the edge of the defect transplanted with HAPLLA than those with PLLA. These results indicate that the HA coating promoted the formation of new bone inside the defects covered with PLLA. It has been reported that mixing HA with polymer materials increases bone compatibility [24-26]. The result was in accordance with the data on MG63 spread in vitro. The earlier bone elongation observed upon HAPLLA seems to be due to the small amount of HA exposed on the surface. In addition, HA coating promoted the absorption of the scaffold. The absorption had started by 2 weeks for HAPLLA, but not for PLLA. Osteoclast-like cells have been recognized to absorb apatite [7] and mineralized collagen I scaffolds [27]. Osteoclast-like cells may induce the mineralization of PLLA in the presence of HA.

The morphology of bony defects in cleft lip and palate is usually complicated, and therefore, it is typically difficult to adapt the shape that is to be fitted into a bone defect. Bioabsorbable synthetic polymers, such as PLLA used in this study, formed into various shapes—fibers, films, cloths, and porous bodies [28, 29]—have been used in surgery for cleft palate and other congenital malformations. Pediatr Res 2008;63:545-551.


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