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In vivo evaluation of composites of PLGA and apatite with two different levels of crystallinity

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Abstract The cortical bone response towards poly(lactide-co-glycolide) (70/30) (PLGA) (70/30)/apatite complex scaffolds with different levels of crystallinity was investigated. Apatite with different levels of crystallinity, Ca-deficient hydroxyapatite (CDHA), which has a low crystallinity, and a mixture of carbonated hydroxyapatite (CHA) and CDHA, which has a higher crystallinity, were prepared from an aqueous mixture of Ca-EDTA complex, H_2O_2, H_3PO_4, and NH_4OH. Two porous PLGA(70/30)/apatite composite scaffolds, composite scaffold **A** (containing low crystallinity CDHA) and composite scaffold **B** (containing the higher crystallinity CHA/CDHA mixture), were prepared. Afterwards, pure porous PLGA and the two composite scaffolds were implanted into the cortical bone of rabbit tibiae for 12 weeks. High-resolution microfocus X-ray computed tomography and histological examinations revealed a better bone response for composite scaffold **A** compared with PLGA and composite scaffold **B**. For composite scaffold **A**, the original bone defect was almost filled with new bone. Quantitative analysis revealed that composite scaffold **A** produced a significantly greater amount of new bone. The present study demonstrated that the level of apatite crystallinity influences bone response. A PLGA/apatite porous composite with a low level of apatite crystallinity shows promise as a bone substitute or scaffold material for bone tissue engineering.

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1 Introduction

Calcium phosphate compounds such as hydroxyapatite and tricalcium phosphate (TCP) are widely used as bone substitutes because of their excellent biocompatibility, osteoconductivity, and bone-bonding properties. However, calcium phosphate compounds have shortcomings in their mechanical properties such as brittleness and insufficient strength.

To overcome the above-mentioned shortcomings of calcium phosphates, composite materials made of calcium phosphates and biodegradable polymers such as poly(lactic) acid, poly(glycolic) acid, and poly(lactide-co-glycolide) (PLGA) have been prepared. The composite materials have been used as bone substitutes, and numerous studies reported better biocompatibility and enhancement of osteoconductivity [1–8]. Regarding apatite content in the composite materials, Petricca et al. [9] investigated the osteoblas-like cell adhesion on the PLGA composite containing 10, 20 and 30 wt% apatite. They found that the composite containing 30 wt% apatite showed the greatest degree of cell adhesion. Shikinami et al. [4] reported that PLA composite containing 30 or 40 wt % unsintered apatite was clinically effective for bone replacement.

Previously, we prepared two types of apatite with different crystallinities and produced a porous composite containing apatite and PLGA copolymer using the casting/particles leaching method [10]. Apatite was prepared through calcium-ethylenediamine tetraacetic acid (Ca-EDTA) complex.

The apatite with low crystallinity was Ca-deficient hydroxyapatite (CDHA), and the apatite with higher crystallinity was a mixture of carbonate hydroxyapatite (CHA) and CDHA. The starting materials for both apatites were the same, i.e. a mixture of Ca-EDTA, H_3PO_4, H_2O_2, and NH_4OH aqueous solution. Different heat and ultrasonic treatments produced the different levels of apatite crystallinity.

In our previous study [10], the degradation of a PLGA/apatite porous composite with a low crystallinity was faster in phosphate buffered saline solution than the degradation of other apatite composites with higher levels of crystallinity. The rate of calcium phosphate deposition on the PLGA/apatite composites after immersion in simulated body fluid (SBF) was also affected by the differences in their crystallinity. The porous PLGA/apatite composite with low crystallinity induced a more rapid and greater amounts of calcium phosphate precipitation. An in vitro human gingival fibroblast assay showed no adverse effects on cell attachment to PLGA/apatite composites.

On the basis of the above-mentioned results, we aimed to evaluate the in vivo cortical bone response towards PLGA/apatite composite scaffolds with different levels of crystallinity. A porous PLGA/apatite composite scaffold was implanted into the cortical bone of rat tibiae. It was hypothesized that the level of apatite crystallinity in the composite scaffold would influence the bone healing process.

2 Materials and methods

2.1 Apatite, PLGA, and porous PLGA/apatite composite scaffold preparation

Apatite with different levels of crystallinity, PLGA and porous PLGA/apatite composite scaffolds, were prepared according to the method of a previous report [10].

The synthesis scheme for apatite with different levels of crystallinity is shown in Fig. 1.

Briefly, CDHA powder with low crystallinity was prepared from a mixture of Ca-EDTA complex, 30% H_2O_2 (Santoku Chemical Industries Co. Ltd., Tokyo, Japan), 85% H_3PO_4 (Taisei Chemical Co. Ltd., Tokyo Japan), and 28% NH_4OH aqueous solution (Taisei Chemical Co. Ltd., Tokyo Japan). The Ca/P ratio was adjusted to 1.67, and the molar ratio of H_2O_2 to Ca was thus adjusted to 12.

The mixture of CHA and CDHA powder, hereafter called CHA/CDHA mixture, was prepared by mixing Ca-EDTA complex, 30% H_2O_2, 85% H_3PO_4, and 28% NH_4OH. The Ca/P ratio was adjusted to 1.67, and the molar ratio of H_2O_2 to Ca was thus adjusted to 25.

The crystal structure of the apatites was characterized by X-ray diffractometry (XRD, MXP-18 AHF22, Bruker AXS, Kanagawa, Japan). The θ–2θ scan mode was

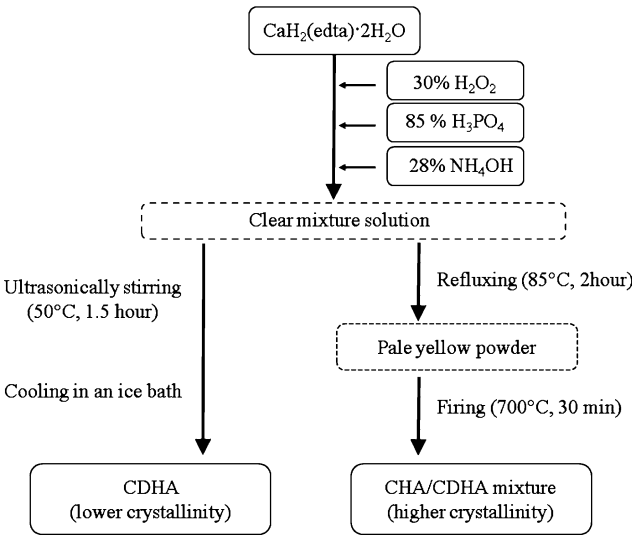

Fig. 1 A schematic drawing of the preparation of apatite with two different levels of crystallinity

employed with CuKα as the X-ray source at 45 kV and 300 mA.

PLGA (70/30: Mw = 11.5 × 10^4) was prepared via ring-opening co-polymerization of L-lactide (PURAC, the Netherlands) and glycolide (PURAC, the Netherlands) at a molar ratio of 70:30 [10, 11].

Porous PLGA/apatite composite scaffolds were manufactured by the solution casting/particles leaching method [10, 12]. Two composite scaffolds, **A** (with low crystallinity: CDHA) and **B** (with higher crystallinity: CHA/CDHA mixture) were prepared. The surfaces of the two composite scaffolds were modified by plasma treatment followed by the collagen anchorage technique as previously reported [10, 13]. The porosity measured using a density bottle was approximately 91–92%.

The porous structure of the composite scaffolds was observed using field-emission scanning electron microscopy (FE-SEM, S-4200, Hitachi, Japan) at an accelerating voltage of 5 kV.

2.2 Implantation procedure

The animal study was conducted in accordance with the animal experimental ethical guidelines of Nihon University School of Dentistry at Matsudo (Certificate Number: ECA-07-0021). Nine 3-month-old female Japanese White Rabbits weighing about 3.5–4 kg were used. Porous PLGA as a control and two PLGA/apatite composite scaffolds, composite **A** and **B,** were inserted into the cortical bone of rabbits according to a previously reported technique [14]. Each animal received two types of porous materials. Namely, three rabbits received composite **A** and composite

B in each tibia, respectively. Other three rabbits received composite **A** and control PLGA, and remaining three rabbits received composite **B** and control PLGA in each tibia, respectively. Thus, the number for each material, composite **A, B** and control PLGA, was six. Before surgery, all implants were sterilized with ethylene-oxide gas.

Surgery was performed under general inhalation anesthesia with a 4% isoflurane and oxygen mixture, which was reduced to 2% isoflurane during surgical manipulation. Local anesthesia was administered by xylocain injection. To reduce the perioperative infection risk, a prophylactic antibiotic, Shiomalin® (equivalent to Latamoxef Sodium, Shionogi & Co., Ltd, Japan), was administered postoperatively by subcutaneous injection.

Porous PLA or PLA/apatite composite scaffolds (4.5 mm × 3.5 mm × 2 mm) were inserted into the left and right tibial diaphyses of the rabbits. For insertion of the porous material, each animal was immobilized on its back. The hind legs of the rabbits were shaved, washed, and disinfected with iodine tincture. A longitudinal incision was made along the medial surface of the left and right tibiae, and the bone was exposed by blunt dissection. Cortical bone defects measuring 2 × 4 mm^2 were created through the medial cortex and the medulla. These defects were prepared with a very gentle surgical technique using a low rotational drilling speed (500 rpm) and continuous internal cooling. After press-fit insertion, the soft tissues were closed in separate layers using restorable Vicryl 3-0 sutures.

Postoperatively, the animals were placed in a standard cage. They were fed with water and rabbit diet ad libitum and were allowed to move unrestricted at all times. The rabbits were sacrificed by peritoneal injection of an overdose of pentobarbital sodium (Nembutal®).

2.3 Micro CT and histological observation

The composite scaffolds and surrounding bone were excised immediately after sacrifice. Then, the excess tissue of the excised specimen was removed and was fixed in 10% buffered formalin solution. Afterwards, containing tissue blocks containing composite scaffolds were dehydrated through a graded series of ethanol and embedded in methylmethacrylate. After polymerization at 37°C, the embedded samples were obtained.

Before preparing the histological sections, the healing condition of the defects in the tibia was first observed using high-resolution microfocus X-ray computed tomography (micro-CT, TOSCANER-30000μhd, Toshiba IT & Control Systems Corporation, Tokyo, Japan) with a voltage of 74 kV, a tube current of 110 μA, a slice thickness of 0.160 mm, and a slice pitch of 0.8 mm. For micro CT observation, three embedded specimens for each material were randomly selected and were observed.

After micro CT observation, non-decalcified thin sections were prepared using a cutting-grinding technique (EXAKT-Cutting Grinding System, BS-300CP band system & 400 CS microgrinding system, EXAKT) [15]. Sections with a final thickness of approximately 50 μm were made in a transverse direction perpendicular to the axis of the implants and were stained with toluidine blue. The bone response towards the composite materials was evaluated using a light microscope (Eclipse E800M, Nikon, magnification ×100).

Besides the histological observations, a histomorphometrical analysis of new bone formation was performed. Four or five sections were prepared from the same composite material and one section was used for histomorphometrical evaluation.

The degree of bone regeneration was calculated as the percentage of new bone area in the graft area, as measured by image analysis software (Image Pro-Plus, Media Cybernetics. Silver Spring, MD, USA). The size of interest region surrounding healing bone was determined as 2 mm × 3 mm, which was slightly smaller than that of inserted composite materials. The rate of new bone formation was defined as the percentage of the area of new bone formed in the region against the area of original bone which was present in the interest region before surgery.

The data were statistically analyzed using one-way ANOVA and Scheffe's test for comparison among the means at $P = 0.05$.

3 Results

3.1 Apatite and PLGA/apatite composite

Figure 2 shows the XRD patterns of the prepared apatite. Both CDHA (a) and the CHA/CDHA mixture (b) showed apatite peaks at 002, 211, 112, 300, and 213. The peaks in the XRD pattern of CDHA were broader than those of CHA/CDHA due to its low crystallinity.

Figure 3 shows FE-SEM observations of porous PLGA/apatite composite scaffold **A** (a), scaffold **B** (b) and control PLGA (c). The porosity of the structures was confirmed for composite **A**, composite **B,** and control PLGA, and the presence of pores of around 200–300 μm in diameter was observed.

3.2 Micro-CT observation

During the test period, the experimental animals remained in good health. At sacrifice, no clinical signs of inflammation or adverse tissue reactions were seen.

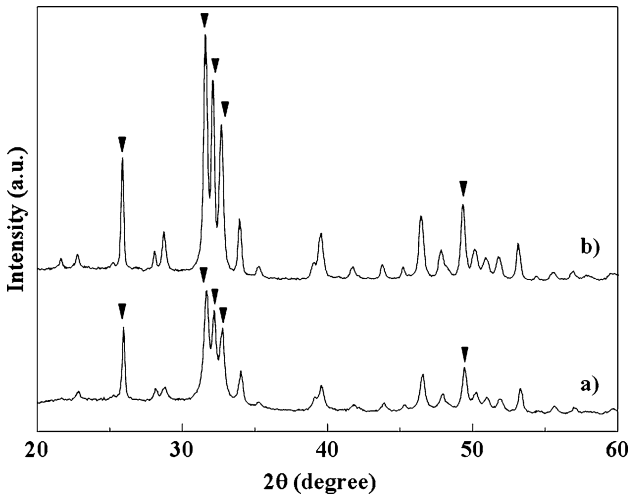

Fig. 2 XRD patterns of apatite prepared from Ca-EDTA. (**a**) CDHA with low crystallinity, (**b**) CHA/CDHA mixture with higher crystallinity. ▼ = apatite. The peaks in the XRD pattern of CDHA were broader than those of CHA/CDHA due to its low crystallinity

Figure 4 shows the micro-CT appearance of the rabbit tibiae after 12 weeks of porous PLGA/HA composite scaffold implantation (**A**: (a), **B**: (b), and control PLGA: (c)). The original defect was almost filled with new bone for composite scaffold **A**. For composite scaffold **B**, the original drill hole in the tibia was partly closed. On the contrary, the original defect was still present and no new bone formation was recognized for the control PLGA.

3.3 Histological observation

The histological appearances of porous PLGA/HA composite scaffolds **A** (a), **B** (b), and control PLGA (c) after 12 weeks of implantation are shown in Figs. 5, 6 and 7. No inflammatory reactions were formed in all samples. The overall cortical bone responses to PLGA and the two PLGA/HA composite scaffolds were clearly different. Their histological appearance corresponded with the results of the micro-CT observation.

For composite scaffold **A** (Fig. 5a), the original bone defect was almost filled with new bone, and no residue of degraded composite was recognized. Bone remodeling was proceeding, and the new bone had almost developed into mature cortical bone. Bone marrow cells (asterisk) were observed beneath the new bone. Apatite crystal formation was clearly observed along the edge of newly formed bone in a higher magnification observation of boxed area (Fig. 5b).

The bone defect for composite scaffold **B** was bridged by new bone but the original drill hole was not completely filled with new bone (Fig. 6a). The composite material was degraded into the particles and degraded particles were dispersed in the bone defect and in the bone marrow area. Some degraded particles were embedded in the bone matrix at the edge of newly formed bone (Fig. 6b). Calcification around the degraded particles was identified by toluidine blue staining.

On the contrary, a little new bone formation was observed at the edge of the original bone hole for the control PLGA specimen, but most of the original bone defect was still present. Implanted PLGA material was completely diminished. In the space of bone defect, connective tissue with fibroblast rich (arrow head in Fig. 7a) and capillary vessel (arrow in Fig. 7b) were observed. Small apatite crystals were identified at the edge of newly formed bone (Fig. 7b).

No clear appearances of macrophages nor foreign-body giant cells recognized in the present histological sections for all specimens.

Table 1 shows the quantitative evaluation of new bone formation. The control PLGA showed a significantly low value, and composite scaffold **A** showed a significantly high value of new bone formation among the porous implanted materials ($P < 0.052$).

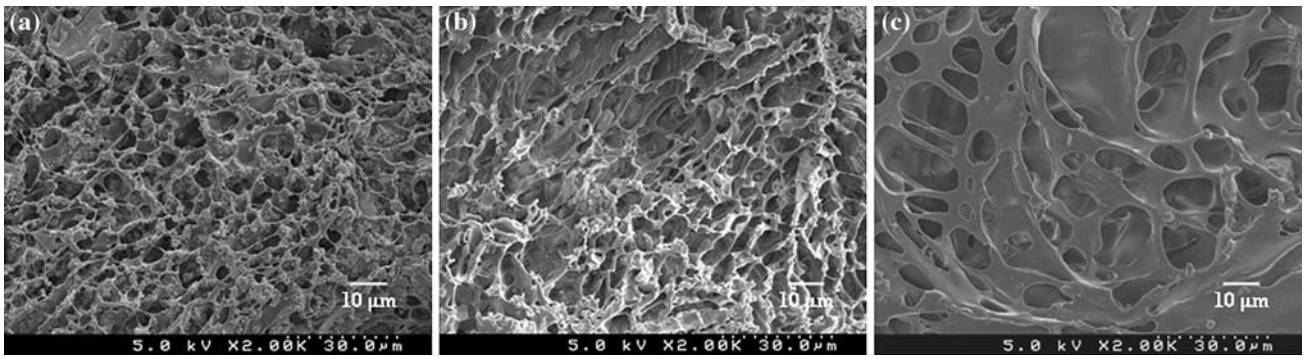

Fig. 3 FE-SEM images of porous PLGA/apatite composite scaffold **A** (**a**), composite scaffold **B** (**b**), and PLGA (**c**). The porosity of the structures was confirmed for composite scaffold **A**, **B**, and pure PLGA, and the presence of pores around 200–300 μm was observed

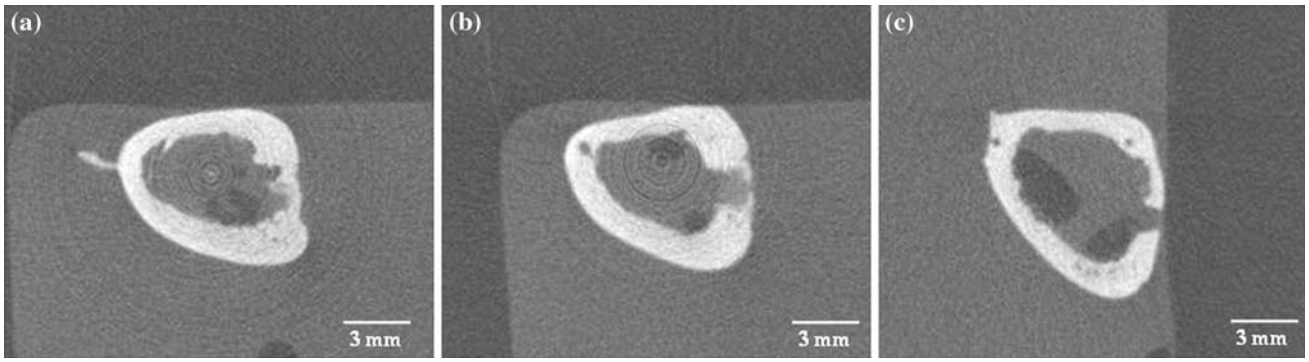

Fig. 4 Micro-CT views of rabbit tibiae after 12 weeks of implantation of porous PLGA/apatite composite scaffold **A** (**a**), composite **B** (**b**) or control PLGA (**c**). The original defect was almost filled with new bone for composite scaffold **A**. For composite scaffold **B**, the original drill hole in the tibia was partly closed. On the contrary, the original defect was still present and no new bone formation was recognized for the control PLGA

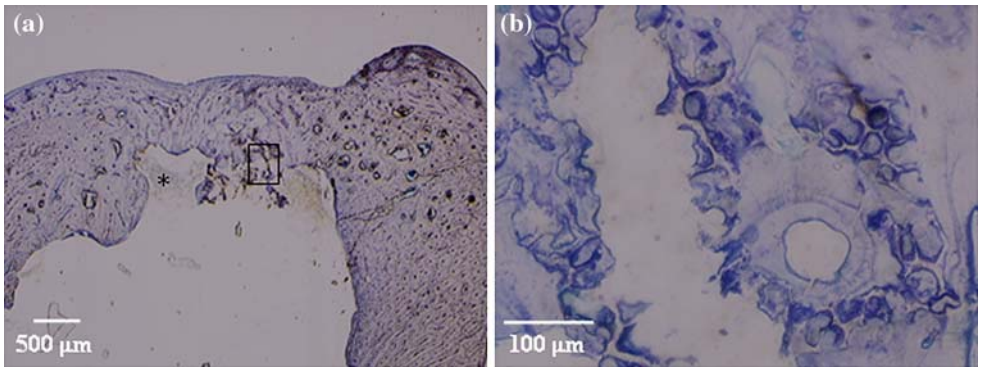

Fig. 5 The histological appearance after 12 weeks of implantation of porous PLGA/apatite composite scaffold **A**. (**a**) Lower magnification of new bone formation area, (**b**) higher magnification of apatite crystal formation. *Asterisk* denotes bone marrow cells. The original bone defect was almost filled with new bone, and no residue of degraded composite was recognized. Bone remodeling was proceeding, and the new bone had almost developed into mature cortical bone. Bone marrow cells were observed beneath the new bone. Apatite crystal formation was clearly observed along the edge of newly formed bone in a higher magnification observation of boxed area (**b**)

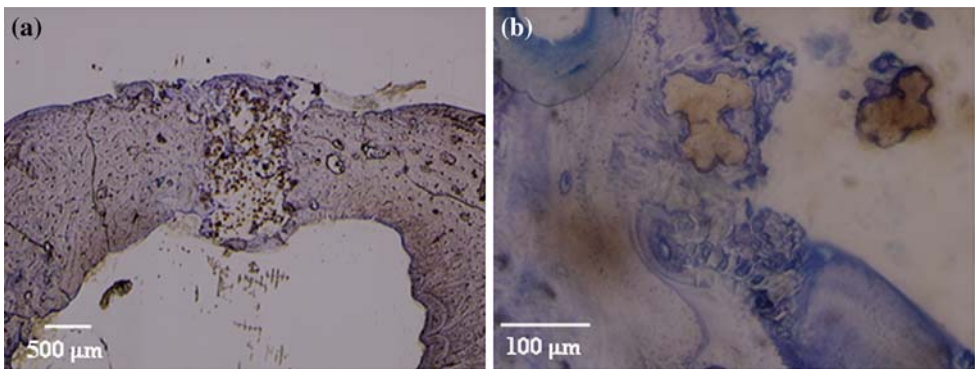

Fig. 6 The histological appearance after 12 weeks of implantation of porous PLGA/apatite composite scaffold **B**. (**a**) Lower magnification of new bone formation area, (**b**) higher magnification of degraded particles. The bone defect for composite scaffold **B** was bridged by new bone but the original drill hole was not completely filled with new bone (**a**). The composite material was degraded into the particles and degraded particles were dispersed in the bone defect and in the bone marrow area. Some degraded particles were embedded in the bone matrix at the edge of newly formed bone (**b**). Calcification around the degraded particles was identified by toluidine blue staining

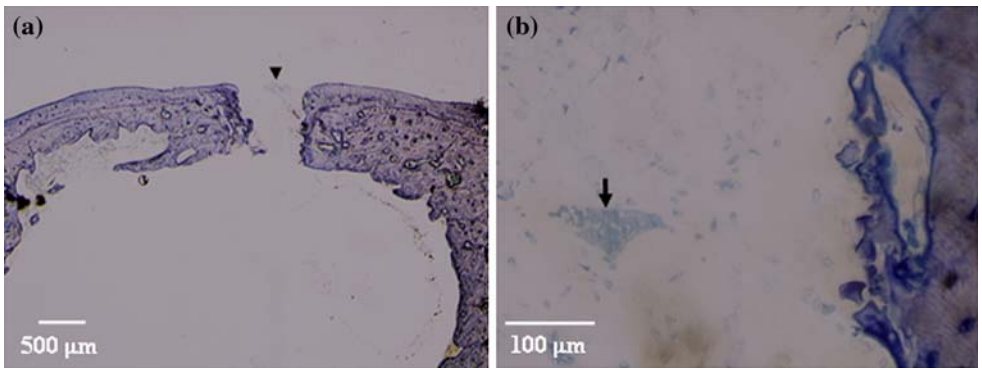

Fig. 7 The histological appearance after 12 weeks of implantation of control PLGA. (**a**) Lower magnification of new bone formation area, (**b**) higher magnification of the edge of newly formed bone. *Arrow* denotes connective tissue with fibroblast rich. *Arrowhead* denotes capillary vessel. A little new bone formation was observed at the edge of the original bone hole for the control PLGA specimen, but most of the original bone defect was still present. Implanted PLGA material was completely diminished. In the space of bone defect, connective tissue with fibroblast rich (**a**) and capillary vessel (**b**) were observed. Small apatite crystals were identified at the edge of newly formed bone (**b**)

Table 1 The percentage of bone regeneration in rabbit cortical bone defects after porous composite implantation

PLGA	Composite scaffold **A**	Composite scaffold **B**
51.4 ± 9.0	87.0 ± 5.4*	71.9 ± 4.2**

$n = 6$

*,** Means were significantly different at $P < 0.05$

4 Discussion

In the present study, we evaluated the cell affinity and bone response towards PLGA/apatite composite scaffolds with different levels of crystallinity.

Druncan and Brown [16] prepared a PLGA/CDHA composite by hydrolyzing α-TCP. They reported that the mechanical properties of PLGA/CDHA composites vary with hydrolysis temperature. In the present study, PLGA/apatite composites were prepared by simple mixing of PLGA and apatite such as CDHA or a mixture of CHA and CDHA.

The advantage of apatite synthesis through Ca-EDTA complex is as follows: (1) the reaction mixture is homogeneous, (2) easy control of the Ca/P ratio provides control of the calcium phosphate content, (3) the crystallinity of calcium phosphate can be controlled by regulating the reaction conditions, i.e., temperature, etc. The Ca-EDTA complex technique also produces a thin adherent biocompatible apatite coating that can be used for titanium [17–19].

Two types of apatite with different crystallinities were prepared from a homogeneous aqueous solution of Ca-EDTA complex and H_3PO_4 as a phosphate source. In both cases, 30% H_2O_2 was adequate as the reaction solvent. The ultrasonic stirring was very useful for synthesizing CDHA powder with a sufficient yield while retaining a low level of crystallinity.

Although PLGA and the two PLGA/apatite composite scaffolds have similar porous structures, their in vivo bone responses were markedly different. The implantation of PLGA alone showed little new bone formation in the cortical bone defect. This reveals that the addition of apatite into the composite enhanced the new bone formation.

Moreover, it also revealed that the level of crystallinity of the incorporated apatite influenced the progress of the bone healing process. Composite scaffold **A**, which has a low HA crystallinity, showed a better bone response than composite scaffold **B**, which has a higher apatite crystallinity. The original bone defect was completely filled with new bone after composite scaffold **A** implantation. A significant difference in new bone formation was recognized between composite scaffolds **A** and **B**.

This was due to the difference of the degradation rate of composite scaffold **A** and **B**. It was very difficult to calculate the precise amount of degraded materials because the degraded particles of composite scaffolds **B** were dispersed into the bone marrow area and were also present in the new bone matrix. Moreover, composite scaffold **A** was almost degraded and no clear remaining was identified. Although there was no quantitative comparison, it was clear that composite scaffolds **A** showed more rapid degradation compared with **B**. Composite scaffolds **A** could be rapidly replaced by bone, but composite scaffolds **B** caused only partly replacement.

The rapid degradation of composite scaffold **A** was caused by the low level of crystallinity of apatite. Low level of crystallinity of apatite is easier to dissolve in vivo.

It is suggested that the greater degree of degradation of apatite and dissolution of calcium ions from composite

scaffold **A** stimulated the activity of osteoblast-like cells and enhanced the bone formation. An alternative speculation was that the faster degradation of the HA and greater dissolution of the Ca ions resulted in a supersaturated condition, which was followed by calcium deposition [20, 21].

There are some reports about the influence of apatite crystallinity on bone response in apatite coated titanium implants. Oh et al. [22] reported that the crystallinity of the HA coating can influence the bone-implant interface and insisted that optimizing apatite crystallinity is necessary for optimal bone response. Xue et al. [23] compared the in vivo responses of dogs between apatite coated. Apatite coated titanium implants with 55 and 98% crystallinity and found that the almost fully crystalline apatite coating exhibited inferior bone bioactivity than the 55% crystalline apatite coating during the early postimplantation period. They described that immediate calcium release from the apatite coating is required for initial bone fixation.

In the present study, we compared two different levels of crystallinity of apatite in PLGA/apatite composite scaffolds. A more detailed study with various levels of apatite crystallinity and other factors such as the Ca/P ratio of apatite will be carried out. Moreover, the apatite of composite scaffold **A** is CDHA, and that of **B** is a mixture of CHA and CDHA. It is possible that the difference in apatite structure influenced the bone response. A detailed study concerning the effects of different apatite structures will also be carried out as our next series of experiments.

The clinical relevance between 72 and 82% bone formation obtained in the present study was not clear. Further investigation for clinical relevance should be conducted.

In conclusion, the present study demonstrated that the level of crystallinity of apatite influences bone response. It is suggested that the PLGA/apatite porous composite scaffold with the low level of crystallinity apatite has promise as a bone substitute or scaffold material for bone tissue engineering.

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