Original

Cortical Bone Response Towards Porous Composites of PLGA and Apatite Prepared from Calcium Complexes

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Abstract: Apatites were prepared by varying the ligands of calcium complexes. Two lower crystallinity apatites were prepared from calcium-iminodiacetic acid (Ca-IDA) or calcium- aspartic acid (Ca-Asp) complexes and one higher crystallinity apatite from Ca-IDA complex. Cortical bone responses towards their PLGA composites including different apatites, namely, PLGA/IDA-HA(lower), PLGA/IDA-HA(higher) and PLGA/Asp-HA(lower), were evaluated after implantation into the cortical bone of rabbit tibiae.

Micro-CT observation showed no distinct differences among the three PLGA/apatite composites after 12 weeks of implantation. The original drill holes were partly closed and formation of primary woven bone was observed. Residue of decomposed PLGA was still observed inside the bone marrow.

The histological appearances of porous PLGA/apatite composite scaffolds after 12 weeks of implantation showed similar overall cortical bone response to different PLGA/apatite composites. The original drill hole was not completely filled with new bone. For PLGA/IDA-HA(higher) composite, the original bone defect was partly filled with new bone. Residue of degraded PLGA and remaining apatite was recognized. Both PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) showed residue of degraded PLGA inside the bone marrow. Remnant lower-crystallinity apatite was not clearly recognized.

In conclusion, apatites with different crystallinity were prepared through Ca-IDA and Ca-Asp complexes. The bone responses towards PLGA/apatite composites with different crystallinity were similar, but the degradation behavior of PLGA/apatite composite was different. It is suggested that the degradation of PLGA in PLGA/apatite composite influences new bone formation in addition to the release and dissolution of calcium from apatite.

Keywords: Apatite, PLGA, Porous composite scaffold, Crystallinity, Cortical bone response

Introduction

Composite materials made from calcium phosphate compounds such as hydroxyapatite and tricalcium phosphate (TCP) and biodegradable polymers such as poly(lactic) acid, poly(glycolic) acid, and poly(lactide-co-glycolide) (PLGA) have been proposed to overcome the shortcomings in the mechanical properties of calcium phosphate such as brittleness and insufficient strength. These composite materials have been used as bone substitutes, and numerous studies reported better biocompatibility and enhancement of osteoconductivity¹⁻⁷⁾.

Few studies have reported the relationship between the crystallinity of apatite and bone formation response in vivo. In our previous work, porous composites of PLGA and apatite with different crystallinities were prepared and evaluated for in vivo and in vitro biocompatibility⁸⁻¹⁰⁾. Two types of apatite, a low crystallinity calcium-deficient hydroxyapatite (CDHA) and a higher crystallinity apatite mixture of carbonate hydroxyapatite and CDHA, were prepared from calcium-ethylenediamine tetraacetic acid (Ca-EDTA) complex. PLGA/apatite porous composite with a lower crystallinity showed faster degradation in phosphate buffered saline solution and more rapid and greater amount of calcium phosphate precipitation in simulated body fluid. Moreover, porous PLGA/apatite composite with lower crystallinity produced a significantly greater amount of new bone after

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ligand	Structural formulae	Stability constant of Ca-complex
IDA	$\text{HN} \begin{cases} \text{CH}_2\text{COOH} \\ \text{CH}_2\text{COOH} \end{cases}$	2.59
Asp	$\begin{array}{c} \text{COOH} \\ \\ \text{H}_2\text{N}-\text{CH} \\ \\ \text{CH}_2 \\ \\ \text{COOH} \end{array}$	1.67
EDTA	$\text{HOOCCH}_2 \text{---} \text{N} \text{---} \text{CH}_2\text{CH}_2 \text{---} \text{N} \begin{cases} \text{CH}_2\text{COOH} \\ \text{CH}_2\text{COOH} \end{cases}$	10.61

Figure1. Structural formulae of IDA, Asp and EDTA and the stability constant of each calcium complex

Figure 2. Schematic drawing of apatite preparation

implantation into cortical bone defects of rabbits⁸⁻¹⁰.

The advantages of apatite synthesis through Ca-EDTA complex are the homogeneous reaction mixture, easy control of Ca/P ratio and easy control of apatite crystallinity by regulating the reaction conditions. However, the apatite with lower crystallinity originating from Ca-EDTA complex was calcium-deficient. Also, a large amount of H₂O₂ was needed for the decomposition of Ca-EDTA complex and the yield for CDHA was very low, approximately 32 %. It is presumed that stability constant of the calcium complex affects the decomposition of complex and the yield of apatite. Easier decomposition of calcium complex with a lower stability constant is expected to produce apatite which is not calcium deficient.

Recently, Mochizuki et al. reported that lower-crystallinity apatite could be prepared by varying the ligands of calcium complexes¹¹. Calcium complexes of iminodiacetic acid (IDA) or aspartic acid (Asp), the stability constants of which are smaller than that of EDTA, were employed. In the present study, two lower crystallinity apatites were prepared from Ca-IDA or Ca-Asp complexes and one higher crystallinity apatite was prepared from Ca-IDA complex. *In vivo* bone responses towards their PLGA composites including different apatites were evaluated after implantation into the cortical bone of rabbit tibiae.

Materials and Methods

Apatite preparation

Apatites were prepared via the Ca-IDA or Ca-Asp complexes. The structural formulations of IDA and Asp are shown in Fig.1 along with EDTA. The stability constants of their Ca complex are also listed. The synthetic scheme for apatites is shown in Fig.2. Three kinds of apatite, IDA-HA(lower), IDA-HA(higher) and Asp-HA(lower) were prepared according to the previous report¹¹.

IDA-HA(lower): lower crystallinity apatite from Ca complexes of IDA

A mixed solution of Ca-IDA complex and H₃PO₄ in water was heated to 90°C with stirring. After addition of NH₄OH to the hot solution, white powders immediately precipitated. The solution was stirred at 90°C for 1 h, and IDA-HA(lower) powder was isolated after the cooling.

IDA-HA(higher): higher crystallinity apatite from calcium complexes of IDA

IDA-HA(lower) powder was heated at 850°C for 0.5 hours, and then IDA-HA(higher) powder was obtained.

Asp-HA(lower): lower crystallinity apatite from calcium complexes of Asp

Figure 3. Micro-CT views of rabbit tibiae after 12 weeks of implantation of porous PLGA/apatite composite.

L-Asp, $\text{Ca}(\text{OH})_2$ and H_2O were mixed, and then H_3PO_4 was added. The obtained Ca-Asp-PO_4 solution was heated to 90°C with stirring. After addition of NH_4OH to the solution, white powders were immediately precipitated, and then the solution was stirred for 1 h at 90°C . Asp-HA(lower) powder was isolated from the reacted solution.

PLGA and porous PLGA/apatite composite preparation

PLGA (70/30: $\text{Mw} = 11.5 \times 10^4$) was prepared via ring-opening co-polymerization of L-lactide (PURAC, the Netherlands) and glycolide (PURAC, the Netherlands) at a molar ratio of 70:30¹².

Three kinds of PLGA porous composites (50-/50 mass%) including different apatites, PLGA/IDA-HA(lower), PLGA/IDA-HA(higher) and PLGA/Asp-HA(lower), were fabricated by the solution casting/particle leaching method as described elsewhere^{8,13}. Porous PLGA composites were kept in a dry vacuum until use.

Implantation procedure

The animal study was conducted in accordance with the animal experimental ethical guidelines of Tsurumi University School of Dental Medicine (No.091). Six 3-month-old female Japanese White Rabbits weighing about 3.5-4kg were used. Porous PLGA/IDA-HA(higher), PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) composites were inserted into the cortical bone of rabbits according to a previously reported technique^{9,10}. Each animal received two types of porous PLGA composites. Namely, two rabbits received PLGA/IDA-HA(higher) and PLGA/IDA-HA(lower) composite in each tibia, respectively. Another two rabbits received PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) composite, and the remaining two rabbits received PLGA/Asp-HA(lower) and PLGA/IDA-HA(higher) in each tibia, respectively. Thus, the number of implants of each material, PLGA/IDA-HA(higher), PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) composite, was four. Before surgery, all PLGA/apatite composites 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 PLGA/apatite composites (4.5mm x 3.5mm x 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 2x4 mm 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 thiamylal sodium (Isozol®) after 12 weeks of implantation.

Micro CT and histological observation

Each PLGA/apatite composite and surrounding bone was excised immediately after sacrifice. Excess tissue of the excised specimen was then removed and fixed in 10% buffered formalin solution. Afterwards, tissue blocks containing PLGA/apatite composites were dehydrated through a graded series of ethanol and embedded in methylmethacrylate. After polymerization at 37°C , the embedded samples were obtained.

Before polymerization, the healing condition of the defects in the tibia was observed using high-resolution microfocus X-ray computed tomography (micro-CT, Latheta, LCT-100, Hitachi-Aloka Medical, Ltd, Tokyo, Japan) with a voltage of 50 kV, a tube current of 1 mA, and a slice thickness of 0.1 mm. For micro CT observation, three dehydrated specimens for each material were randomly selected and 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)¹⁴. Sections with a final thickness of

Figure 4. The histological appearance after 12 weeks of implantation of porous PLGA/ IDA-HA(higher) composite
*: remnant apatite

Figure 5. The histological appearance after 12 weeks of implantation of porous PLGA/ IDA-HA(lower) composite.
Arrow: apatite has dissolved

Figure 6. The histological appearance after 12 weeks of implantation of porous PLGA/ Asp-HA(lower) composite
Arrow: apatite has dissolved

approximately 50 μm were made in a direction parallel to the long axis of the implanted composites and were stained with methylene blue and basic fuchsin. The bone response towards the composite materials was evaluated using a light microscope (BX51, Olympus Corp., magnification $\times 100$).

Results

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

Figure 3 shows the micro-CT appearance of the rabbit tibiae after 12 weeks' implantation of porous PLGA/HA composite scaffold implantation. No distinct differences were observed among the three PLGA/apatite composites. The original drill holes were partly closed and formation of primary woven bone was observed. Residue of decomposed PLGA was still observed inside the bone marrow.

The histological appearances of porous PLGA/apatite composite scaffolds after 12 weeks of implantation are shown in Figures 4-6. No inflammatory reactions were seen in any samples,

and no clear appearances of macrophages or foreign-body giant cells were recognized in any specimen. The overall cortical bone responses to the different PLGA/apatite composites were similar and their histological appearance corresponded with the results of the micro-CT observation.

For PLGA/IDA-HA(higher) composite, the original bone defect was partly filled with new bone, and residue of degraded PLGA was recognized inside the bone marrow (Fig. 4a). Higher magnification clearly identified the remnant higher-crystallinity apatite (asterisk) with along with the new bone (Fig. 4b).

The original drill holes were also not completely filled with new bone for both PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) (Figs. 5&6). Residue of degraded PLGA was clearly observed inside the bone marrow and the part where apatite had disappeared was clearly identified (arrow head). Especially, PLGA/Asp-(lower) showed the original PLGA composite shape (Fig. 6). Remaining lower-crystallinity apatite with was not clearly recognized for either PLGA/apatite composite, even under higher magnification.

Discussion

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

The apatite employed in the present study was prepared from Ca complex with IDA or Asp. Previous studies reported apatite synthesis from Ca-EDTA complex. Ca-IDA and Ca-Asp complex have smaller stability constants than Ca-EDTA complex, as shown in Fig.1. Ca-IDA and Ca-Asp complexes could provide higher yields of apatite than Ca-EDTA complex and could produce apatite which was not calcium-deficient.

There are few reports about the influence of apatite crystallinity on bone response. Regarding apatite-coated titanium implants, it was reported that an apatite-coated titanium implant with 98% crystallinity exhibited inferior bone bioactivity to a 55% crystalline apatite coating¹⁵. It is speculated that the release and dissolution of calcium from apatite stimulate osteoblast-like cells and enhance bone formation. An alternative speculation is that the release and dissolution of calcium of apatite will produce a supersaturated condition and provide calcium deposition^{16,17}. In contrast, Pathi et al. prepared apatite nanoparticles with a narrow size distribution and varying crystallinity and suggested that low-crystallinity apatite nanoparticles promote greater adsorption of adhesive serum proteins and enhance breast tumor adhesion while more crystalline apatite nanoparticles stimulate enhanced expression of the osteolytic factor interleukin-8¹⁸.

Our previous studies^{9,10} demonstrated that the level of apatite crystallinity influences bone response, and the PLGA/apatite composite with a lower level of apatite crystallinity produced a significantly greater amount of new bone. Cortical bone defects

of rabbit tibia were almost completely filled with new bone. However, the present study showed no distinct differences of bone formation between porous composites of PLGA and apatites with different levels of crystallinity.

The degradation behaviors of PLGA/apatite composites were different between lower- and higher-crystallinity apatite. Remnant apatite was clearly recognized for PLGA/IDA-HA(higher) composite, while apatite was dissolved for PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) composites. Lower-crystallinity of apatite is easier to dissolve in vivo. It is presumed that the dissolution of apatite is faster than the degradation of PLGA for PLGA/IDA-HA(lower) and PLGA/Asp-HA(lower) composites. Although the detailed mechanism of bone formation is not still clear, it is possible that the faster dissolution of apatite with lower crystallinity caused the insufficient bone formation of PLGA/apatite composite.

The balance between the release and dissolution rate of calcium and bone formation rate is the key factor for the bone healing process. The dissolution of calcium is controlled by the crystallinity of apatite, as mentioned above. Moreover, the release of calcium from apatite is influenced by the degradation of PLGA. Remnant PLGA was clearly identified and PLGA had not completely decomposed after 12 weeks of implantation. These results differed from our previous experiments^{9,10}. In the present study, remnant PLGA might have inhibited new bone formation. The relationship between the degradation behavior of PLGA and bone formation will need to be further investigated in detail.

In the present study, two lower crystallinity apatites were prepared using different ligands, but no clear differences in bone response were observed. The content of apatite in a PLGA/apatite composite will also influence the bone formation. More content of lower crystallinity apatite or less content of higher crystallinity apatite is expected to promote the bone healing process and enhance the new bone formation.

In conclusion, apatites with different crystallinity were prepared through Ca-IDA and Ca-Asp complexes. The bone responses towards PLGA/apatite composites with different crystallinity were similar, but the degradation behaviors of PLGA/apatite composites were different. It is suggested that the degradation of PLGA in PLGA/apatite composite influences new bone formation.

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