The In Vitro and In Vivo Biodegradable Behavior of Hydroxyapatite Granules in the Presence of Different Crystals

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Abstract: The in vitro and in vivo degradation behavior and osteogenic ability of new hydrothermal hydroxyapatites (HAs) were investigated. Two different HA granules with short and long-rod HA crystals (SRHA and LRHA, respectively) were hydrothermally synthesized. A low pH immersion test was performed to observe morphological degradation features. At 2 days, HA in a low pH immersion fluid revealed pitfall-like degradations on the surface. Both HA granules were implanted in rat calvaria bone defects. The biodegradable behavior and effects of new bone formation were histologically and radiographically investigated. At 4 and 24 weeks after surgery, defect areas were filled with implanted HAs and newly formed bone. No differences in boundary area shapes between SRHA and LRHA were found. The area of LRHA degradation was greater compared with SRHA (p < 0.05). At 24 weeks, implanted HA granules showed inward incursion of fibrous tissue and tissue fluid. Accordingly, in vivo hydrothermal HA indicated good osteogenesis and progress.

Key words: hydrothermal hydroxyapatite, biodegradation, bone formation, animal experiment

Introduction

Many artificial synthetic materials, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), have been developed as alternatives to autologous bone-grafting for the repair of large bone defects. Hydroxyapatite is known to adhere to bone without the intervention of soft tissue; however, due to a lack of bioabsorbability, it continues to exist for an extended time without being converted to bone, causing secondary infections, fractures, and other complications. The material itself should be absorbed in vivo and be converted to bone at the same time as the absorption. Although β-TCP is bioabsorbable, it is prone to early resorption, which limits clinical bone regeneration treatment. Additionally, α-TCP is also bioabsorbable and has been used as a calcium phosphate cement (CPC). However, CPCs do not harden on contact with blood, which can lead to loss of cement; it was also been reported that unhardened particles can cause inflammation.

Recently, Ioku et al. succeeded in preparing biodegradable HA from β-TCP using hydrothermal synthesis. This hydrothermal HA (hHA) is characterized by an aggregation structure of rod-shaped HA crystals and low Ca/P ratio, due to a degree of calcium deficiency in the crystals. The porosity of hHA was 60%–80% in an aggregation structure of rod-shaped HA crystals. In a rabbit implant model, an implanted cylindroid block of hHA revealed bone absorption and replacement. The shape of the HA rod crystals may influence various structural and chemical properties. Additionally, the structure of the rod shape may control the degradation rate and the promotion of bone formation, in addition to determining bone biocompatibility of the fine rod structures for use in clinical applications.

In this study, we prepared two different HA granules, which were composed of short and long-rod HA crystals (SRHA and LRHA, respectively) showing the different Ca/P and aspect ratios of the rod-shaped crystals by coordinated application of hydrothermal synthesis. These HA granules were investigated using in vitro and in vivo absorption to confirm the ability of both long-term persistence and early absorption. The present research will enable the establishment of bone graft materials based on new concepts such as accelerated absorption and by enhancing the replacement of bone tissue.

Materials and Methods

Preparation of hydroxyapatite granules

The HA granules were prepared using a hydrothermal method. The materials used for the preparation were α-TCP powder (Taihei Chemical Industrial Co., Japan) and gelatin (Wako Pure Chemical Industrial, Ltd. Japan). Gelatin was used to maintain the shape of the granules during cooling and to render the granules porous after heating. Furthermore, 1.0 g of α-TCP powder was mixed with 5.0 ml of an aqueous gelatin solution (10wt%). The slurry mixture was dropped into 200 ml of stirred (300 rpm) vegetable oil at 80°C, and the oil was stirred for 10 min. The oil was then cooled to 4°C with continuous stirring. The resultant granules were washed with cooled ethanol, filtered, and air-dried at 4°C. To remove gelatin and to retain the crystal phase of α-TCP, they were heated at 1,200°C for 30 min.

Subsequently, to obtain a calcium-deficient composition and to control the aspect and Ca/P ratios, two types of absorbable HA were granu-
mass density of pure HA from mass $M$ and volume $V$ of well-defined samples, based on the measurements. Moreover, HA biomaterial porosity is typically calculated using $\beta$-TCP, respectively.

Well-separated HA and $\beta$-TCP peaks at $2\theta = 31.8^\circ$ and $31.0^\circ$ for HA and $\beta$-TCP were calculated, using the integrated peak intensities of the $\beta$-TCP by heating at $900^\circ$C. After heating, the mass fraction of each HA ratio was calculated by XRD analysis, as reported by Ishikawa et al. [11, 12].

Calcium-deficient hydroxyapatite decomposed to stoichiometric HA and $\beta$-TCP, respectively.

Characterization of samples

The microstructure of the 300–500 μm SRHA and LRHA was analyzed by scanning electron microscope (SEM; SU8000, HITACHI, Tokyo, Japan). To determine the aspect ratios of the rod-shaped crystals of SRHA and LRHA granules, the SEM images of the granular surface (magnification: ×3,000) were used in NIH ImageJ ver. 1.42q software. The length and width of the prolonged c-axis HA of 30 individual crystals were measured to calculate the average aspect ratio.

The SRHA and LRHA were analyzed using powder X-ray diffraction with graphite-monochromatized CuKα radiation, operating at 40 kV and 40 mA (XRD; RINT2000V, Rigaku, Tokyo, Japan). The Ca/P ratio was calculated by XRD analysis, as reported by Ishikawa et al. [11, 12]. Calcium-deficient hydroxyapatite decomposed to stoichiometric HA and $\beta$-TCP by heating at 900°C. After heating, the mass fraction of each HA and $\beta$-TCP were calculated, using the integrated peak intensities of the well-separated HA and $\beta$-TCP peaks at $20 = 31.8^\circ$ and $31.6^\circ$ for HA and $\beta$-TCP, respectively.

The SRHA and LRHA granular specific surface areas were analyzed by nitrogen adsorption in a Brunauer–Emmett–Teller (BET) analyzer (Autosorb-1, Quantachrome Instruments, Florida, USA). All the samples were degassed at 200°C for 120 min prior to nitrogen adsorption measurements. Moreover, HA biomaterial porosity is typically calculated from mass $M$ and volume $V$ of well-defined samples, based on the mass density of pure HA [13], $\rho_{HA} = 3.16$ g/cm$^3$, and porosity = 1-$M$/($V\rho_{HA}$).

The SRHA and LRHA granules (300–500 μm in diameter, 3.0 mg) were immersed in a 1.0 ml acetate buffer (pH 5.0) for 24, 48, and 72 h ($n = 6$); this simulated the low pH environment caused by inflammation in a living body. The calcium ions released in the solution were determined by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8800, Agilent Technologies, California, USA).

Animals and operative procedures

Twenty-four male Wistar rats (12 weeks old; SLC Corporation, Hamamatsu, Shizuoka, Japan) were used in this study; they were kept under standard laboratory conditions, with light–dark-cycling and relative humidity. The principles of laboratory animal care and national laws were followed. The Animal Research Committee of Tohoku University approved all procedures (Approval number: 22MTA-17). The rats were divided into the following two groups: 1) SRHA-implanted group ($n = 12$); 2) the LRHA implanted group ($n = 12$). Six rats in each group were sacrificed at 4 or 24 weeks after implantation.

The rats were anesthetized using intraperitoneal sodium pentobarbital (50 mg/kg of body weight) after a short-time induction of ether inhalation. After subcutaneous injection of 2% lidocaine (with epinephrine 1:80,000) at the surgical site, a 15-mm-long skin incision was made along the midline of the head, and the periosteum was incised along the temporal muscle attachment line and peeled back like a flap. A full-thickness standardized trephine defect with a 8.8 mm diameter was made in the parietal bone under continuous saline irrigation. Extreme care was exercised to avoid injury to the midsagittal blood sinus and dura mater. Then, 20 mg of the 300–500-μm-diameter SRHA or LRHA granules were implanted into the bone defect. After implantation, the flapped periosteum and skin flaps were repositioned and sutured using absorbable thread.

Radiological and histological analyses

The rats were sacrificed by an overdose intraperitoneal injection of sodium pentobarbital (150 mg/kg) at 4 and 24 weeks after surgery, and they were fixed with a 10% buffered formaldehyde by perfusion through the aorta. The calvarial samples were dissected from the sacrificed animals and scanned using a micro-computed tomography (CT) system (Xmate-E090; Comscantechno Company Ltd., Yokohama, Japan). The exposure parameters were 80 kV and 75 μA, and the distance from the X-ray source to the sample stage was 100 mm. The three-dimensional (3D) images were reconstructed on a computer using 3D structural analysis software (TRI/3D-BON; Ratoc System Engineering Company, Ltd., Tokyo, Japan).

After micro-CT scanning, all the samples were resected in the coronal plane, kept in 70% ethanol overnight at 4°C, and decalcified in 10% ethylenediaminetetraacetic acid in a 0.01 M phosphate buffer, pH 7.4, for 2 weeks at 4°C. The samples were dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections of 6-μm thickness were prepared and stained with hematoxylin and eosin (H&E). Photographs were taken using a photomicroscope (BX-51; Olympus Corporation, Tokyo, Japan).

Histomorphometric analyses

Histomorphometric analyses were performed at 4 and 24 weeks after surgery using tissue specimens stained with H&E, using NIH ImageJ. The gross area of HA granules remaining in the body was measured as the sum of the areas inside the outline of HA granules. The areas of tissue fluid and cells’ penetration into HA granules were measured as the
area stained with H&E. Moreover, the ratio of the area of tissue fluid and cell penetration into HA granules to the gross area of HA granules was calculated.

**In vitro and in vivo evaluation of the absorption mechanism of biodegradable hydroxyapatite in a low pH environment**

As an *in vitro* experiment, larger SRHA or LRHA granules (800–1,000 μm diameter) were used for observation to prevent the shape from collapsing after dissolution and presenting observational difficulties. The SRHA or LRHA granules were immersed in an acetate buffer (pH 5.0) for 1 or 2 days; this simulated the low pH environment caused by inflammation in the living body. Morphological changes in the HA granular surface after 1 and 2 days were observed using SEM.

Moreover, to evaluate *in vivo* absorbability, 800–1,000-μm-diameter HA granules were implanted into defects 3.8 mm in diameter, introduced into rat calvaria. Four male Wistar rats (25 weeks old) were anesthetized. In this experiment, as an intra-individual right–left comparison, two full-thickness standardized trephine defects of 3.8 mm in diameter were made in the parietal bone on both the right and left side. Then, 800–1,000-μm SRHA or LRHA granules were implanted into the bone defects. Four weeks after surgery, rats were sacrificed by an overdose of anesthetic and fixed using 4% paraformaldehyde.

The calvarial samples were dissected from the sacrificed animals and attached to an acryl board with resin. The regions, including the granules, were cut out with a low-speed diamond wheel saw (SBT650, South Bay Technology, California, USA) and dehydrated in ethyl alcohol. The samples were subjected to freeze-drying using a t-BuOH Freeze Dryer (VFD-21S, Vacuum Device Inc., Ibaraki, Japan). Degradation, localization, change in the crystal structure, and cell penetration were observed using SEM.

**Statistical analyses**

Statistical analyses were performed using two-way analysis of variance (ANOVA). Furthermore, ANOVA was used after confirming the homogeneity of the sample using Bartlett’s test. Tukey’s multiple comparison test was followed by ANOVA. Significance was inferred for $p < 0.05$.

**Results**

**General sample features**

Rod-shaped crystals from spherical HA granules were observed by SEM. The SRHA comprised rod-shaped crystals approximately 5.5 μm in length, and LHRA comprised rod-shaped crystals approximately 11.3 μm in length (Fig. 2). The aspect ratio of SRHA was 11 and that of LRHA was 42 (Table 1). Using XRD analysis, no phase other than HA was detected for SRHA and LRHA (Fig. 3). These results showed that both SRHA and LRHA were pure and uniform HA. The XRD measurements also showed Ca/P ratios of 1.58 and 1.53 for SRHA and LRHA, respectively. The specific surface area analysis by nitrogen adsorption in a BET analyzer showed surface areas of 2.6 m$^2$/g and 3.6 m$^2$/g for SRHA and LRHA, respectively. No differences in porosity between

![Figure 2. SEM images of the surface of SRHA granules (a, b) and LRHA granules (c, d). Both SRHA and LRHA granules have spherical shapes with same diameters (a, c, magnification: ×200). SRHA was composed of short rod-shaped crystals (b) and LRHA was composed of longer rod-shaped crystals than SRHA (d). (magnification: ×3,000)](image-url)
SRHA and LRHA granules were found (Table 1). The Ca concentration dissolved in an acetate buffer (pH 5.0) of SRHA and LRHA determined by ICP-MS showed that LRHA dissolved more rapidly than SRHA within 24, 48, and 72 h (Fig. 4).

**Radiological and histological analyses**

Regarding the evaluation of osteogenesis at 24 weeks after surgery in critical-size defects by micro-CT, no clear differences were observed between the SRHA and LRHA groups (Fig. 5). In histological observations, the HA granules of the LRHA group showed a greater infiltration than those of the SRHA group; this was determined by observing the fibulin-like extracellular matrix and inflammatory cells at 4 and 24 weeks after surgery (Fig. 6). At 24 weeks, the HA of both groups showed the ability to induce bone development inside and outside the granules (Fig. 6). Using radiological and histological observations, bone-like tissue that formed around the HA granules was considered bone tissue. Histological evaluation showed that the absorption of HA granules was more advanced; here, tissue fluid and cell penetration into granules were also observed more frequently in the LRHA group than in the SRHA group. Tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multi-nucleated giant cells were present around granular surfaces at 4 and 24 weeks after implantation. These cells were seldom observed inside the HA granules (Fig. 7).

**Histomorphometric analyses**

Histomorphometric analyses were performed at 4 and 24 weeks after surgery using H&E-stained tissue specimens and NIH ImageJ (Fig. 8). The areas of tissue fluid and cell penetration into HA granules at 4 and 24 weeks after surgery were significantly larger in the LRHA group than in the SRHA group (Fig. 8a); however, no significant differences in the size of the areas between 4 and 24 weeks within the same group occurred (Fig. 8a). Furthermore, the gross area of HA granules remaining in the body showed no significant differences between the two groups at 4 and 24 weeks after surgery (Fig. 8b). The ratio of the area of tissue fluid and cell penetration into the HA granules to the overall area of HA granules was significantly larger at 4 and 24 weeks in the LRHA group compared with the SRHA group.

**In vitro and in vivo evaluation of the absorption mechanism of biodegradable hydroxyapatite in a low pH environment**

As an in vitro evaluation of a biodegradable HA absorption mechanism, the acetate buffer immersion test confirmed that the dissolution of the rod-shaped structure of the HA surface proceeded further over time in the LRHA group than in the SRHA group. According to the dissolution of the rod-shaped structure, larger and more micropores formed on the HA surface in the LRHA group than in the SRHA group (Fig. 9).

To perform the in vivo evaluation for absorbability of the 800–1,000-μm HA granules, the granules were surrounded with connective tissue, and cells were observed around the micropores. Larger and more micropores formed on the HA surface in the LRHA group than in the SRHA group. More cells were observed in the micropores in the LRHA group than in the SRHA group (Fig. 10).
Figure 6. Histological sections stained with hematoxylin-eosin. A section of SHRA group at 4 weeks is shown in a and that of 24 weeks is shown in b. A section of LRHA group at 4 weeks is shown in c and that of 24 weeks is shown in d.

Figure 7. Histological sections stained with tartrate resistant acid phosphatase (TRAP). A section of SHRA group at 4 weeks is shown in a and that of 24 weeks is shown in b. Red colored cells: TRAP-positive osteoclastic multi-nucleated giant cells. A section of LRHA group at 4 weeks is shown in c and that of 24 weeks is shown in d. Red colored cells: TAP-positive osteoclastic multi-nucleated giant cells.
Figure 8. The area of the penetration of tissue fluid and cells (a), the gross area of HA granules remained in the body (b), and its ratio (c=a/b×100).

Figure 9. SEM images of HA granules before immersion in acetate buffer (a-d) and after immersion in acetate buffer (pH 5.0) for 1 day (e-h) and 2 days (i-l). SRHA granules are shown in a, b, c, e, i, and j, and LRHA granules are shown in c, d, g, h, k, and l. Some micropores (arrows) were formed on the surface of HA granules after immersion in acetate buffer. (magnification: ×100, ×500)
Discussion

The effects of morphological and chemical physicochemical features on the absorbability of hydroxyapatite

Recently, HA with biodegradability has been reported as suitable for use as artificial bones. The absorbability of HA is believed to be affected by morphological and physicochemical features, including porosity, aspect ratios, Ca/P ratios, and surface area. During the manufacturing process phase, the pH value of the starting reaction solution and the temperature of hydrothermal treatment were shown to be the most significant variables for altering HA structure and morphology. In this study, two types of HA granules with different Ca/P ratios and the aspect ratios of rod-shaped crystals showed different absorbability in vitro and in vivo experiments. Larger porosity, lower Ca/P ratio, and larger surface areas were believed to favor HA absorbability. Thus, HA absorbability can be designed by controlling various conditions of hydrothermal treatment.

The biocompatibility of biodegradable hydroxyapatite by the application of hydrothermal synthesis

In general, HA, which comprises the principal inorganic component of bones and teeth, is believed to exhibit high biocompatibility with bone. However, hydrothermally synthesized HA is a micro-structured material surrounded by many HA crystal rods. It is known that sodium urate needle crystals, or the needle structures of carbon nanotubes or asbestos, stimulate the surrounding tissue, and cause inflammation. Thus, the biological safety of HA was required to be evaluated. Radiological and histopathological findings showed no pathological changes such as bone resorption or inflammatory reactions around the HAs. Accordingly, these new HAs were considered to be biocompatible with bone.

In vivo biodegradation of hydrothermal hydroxyapatite

In this study, hydrothermal HA was designed as an absorbable biomaterial by controlling the features of the HA crystals. However, to date, its absorption behavior in vivo has not been investigated in detail. In vivo biodegradation of hydrothermal HA appeared not only predominantly on the surface of granules, but also inside the granules. Although HA absorption was considered to have been caused by osteoclasts during the early phase of HA implantation, osteoclasts were seldom observed at the margin and inside the HA granules, although they were often observed in the periphery of neonatal bone. The surface of granules exhibited more osteoclastic TRAP-positive cells, which were surrounded by newly formed bone and revealed no marked absorption. Our results may indicate that a low pH environment, in addition to TRAP-positive cells, influenced hydrothermal HA absorption.
In vitro evaluation of the biodegradable hydroxyapatite absorption mechanism in a low pH environment

It was suggested that HA granule absorption was caused by the surrounding low pH environment. The implanted HA was exposed to a low pH environment because of the inflammatory environment associated with surgical invasion. In experiments in which granules were immersed in a buffer solution of pH 5.0, HA crystal dissolution was confirmed. We hypothesized that the local pH decreased because of acute inflammation, which occurred immediately after implantation caused the dissolution of the granules. However, as a result of the buffering capacity of the tissue fluid after inflammation, pH did not affect HA absorption over extended periods. It was believed that the pH decrease, which was required for the dissolution of HA crystals, was caused not only by the inflammatory response, but also by the phosphoric acid by-product that formed during hydrothermal synthesis in the HA granules. In addition, inflammatory cells may affect HA absorption. When cells attached to the hollow formed by inflammation, pH was reduced locally by cellular mechanisms. The pH decrease caused the formation of a deeper hole, and cells entered the interior of the granules. Thus, cells invaded the granules internally and formed a larger cavity. Then, osteoprogenitor cells entered the cavity and differentiated into osteoblasts to form bone-like tissue.

The influential factor of biodegradation

It has been well-established that a calcium phosphate-containing bone matrix is absorbed by acids. Our results showed a significant difference in the rate of LRHA and SRHA degradation between direct absorption by acid-secreting osteoclasts and indirect absorption in the surrounding low pH environment. The LRHA and SRHA were characterized by differences in aspect ratio and Ca/P ratio. As the specific surface area increased due to the high aspect ratio, the area exposed to the acid increased. However, it is also known that calcium phosphate generally increases its absorbability as the Ca/P ratio decreases. As such, it was believed that LRHA and SRHA with different aspect and Ca/P ratios indicated different absorption rates. This suggested that HA can be clinically applied as an HA bone substitute for adjusting bone replacement rate.

Conflict of Interest

The authors declared that no COI exists.

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