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Control of Bisphosphonate Release Using Hydroxyapatite Granules

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Running Heads: Control of bisphosphonate release using hydroxyapatite granules

Abstract: The efficacy of hydroxyapatite (HAp) as a carrier was investigated in order to establish a method of local administration of bisphosphonates (Bps), which has currently been administered systemically. HAp granules 300-500 μm in size were prepared with different physicochemical features by altering the sintering temperature. To ascertain the physicochemical properties of the HAp granules, their crystallinity was assessed using X-ray diffraction, the surface morphology was examined under scanning electron microscopy, and the specific surface area and calcium dissolution were evaluated. Different Bps-HAp composites were subsequently prepared and the concentration of Bps released from these composites was measured. The influence of Bps-HAp composites on the rate of osteoclast survival was also evaluated. The results revealed that 1) HAp solubility depends on the sintering temperature; 2) The concentration of released Bps could be controlled by regulating the sintering temperature of HAp as a carrier; and 3) Bps released from Bps-HAp composites reduced the number of osteoclasts. These findings indicated that Bps-HAp composites could be locally administered as a drug delivery system to areas with bone resorption.

Keywords:

35.00 bone remodeling

77.00 dental/endosteal implant

81.00 drug delivery/release

134.00 hydroxy(1)lapatite

142.00 in vitro

bisphosphonate

bisphosphonate, osteoclast, hydroxyapatite, drug delivery system, bone regeneration

INTRODUCTION

Bisphosphonates (Bps) have been used as an effective drug for patients suffer from osteoporosis. The Bps chemically binds to the calcium phosphate in the bone and suppresses the activation of osteoclasts. In addition, Bps are known to inhibit bone resorption by directly facilitating osteoclast apoptosis [1, 2].

In dental implant and maxillofacial therapy, two methods for the local administration of Bps have been investigated. One method is the use of Bps to immobilize the implant in order to improve bone quality around the implant [3-6]. Yoshinari et al. and Kajiwara et al. reported that the Bps-immobilized titanium implants had a higher percentage of bone contact than those that were not immobilized, suggesting that the immobilization of Bps was effective in the promotion of osteogenesis [4, 7]. The other method is a drug delivery system (DDS) in which a carrier is used to deliver Bps to bone-deficient areas. However, the carrier must be biocompatible and have an affinity for Bps. Local administration based on a DDS uses a carrier that can store a drug in the body. Consequently, changes in the biological environment result in the release of the drug from the carrier to express pharmacological effects.

Biodegradable polyesters such as poly glycolic acid derivatives have been investigated as carriers of Bps [8,9]. Calcium phosphates have been examined as carriers in DDS [10,11]. Of the different forms of calcium phosphate, hydroxyapatite (HAp) appears to be the most promising candidate because HAp is controllable a wide rage of solubility with a following reason [12]. Compared to ß-tricalcium phosphate, HAp has a lower degree of solubility under a high degree of crystallinity. The crystallinity can be reduced by lowering the sintering temperature while the solubility can be increased, enabling control within a wide range of levels of HAp solubility and Bps release. Since the solubility of HAp is affected by pH, bone resorption facilitated by inflammation may allow the creation of an environment in which Bps can be more easily released from a carrier. In addition, HAp has a high affinity to Bps and, therefore, should not hinder the pharmacological activity of Bps based on the binding mechanism involved [2]. Thus,

the release of Bps could be controlled by regulating the solubility of HAp.

The present study aimed to examine control of the concentration of Bps released from HAp as a DDS. We synthesized HAp and its composite combined with Bps. The obtained HAp and composite displayed the characteristics of HAp as a carrier. After the Bps-HAp composites were prepared, the concentration of Bps released was measured. Furthermore, whether the Bps that was released from the composite was capable of suppressing osteoclasts was investigated.

MATERIALS AND METHODS

Sample Preparation

HAp granules as carriers

HAp powder was synthesized by the wet method using calcium hydroxide (99.9%, Wako chemical, Japan) and phosphoric acid (purity 85.0%+, Wako chemical, Japan) as starting materials, then dried at 110°C. The powder was sintered to form granules at 400°C or 800°C for 3 hours or at 1200°C for 1 hour with a heating rate of 5°C/min (HAp400, HAp800, and HAp1200, respectively). The sintered materials were ground and sieved to select the 300-500 μm particles. The granules used in the present study were prepared by Mitsubishi Materials (Saitama, Japan).

Bps-HAp composites

Pamidronate (disodium 3-amino-1-hydroxypropylidene-1,1-bisphosphonate pentahydrate, Ciba-Geigy, Japan) was used for the Bps. A 10-mM Bps solution was prepared by dissolving 30 mg of pamidronate in 10 ml of distilled water. 10 mg of HAp400, HAp800, or HAp1200 was soaked in 1 ml of the Bps solution at 37°C for 24 h to load the Bps into the HAp carriers. After the solution was filtered, the remaining granules were gently washed in distilled water to remove any Bps that was physically adsorbed. The prepared Bps-HAp composites were referred to as Bps400, Bps800, and Bps1200, according to the sintering temperature of the

carrier HAp granules.

Characterization of HAp Granules

Crystallinity by X-ray diffraction

An X-ray diffractometer (RINT2500, Rigaku, Japan) was used to assess the crystallinity of the HAp granules. The X-ray source was Cu-Ka1, and X-ray diffraction was performed at 50 kV and 200 mA. While ascertaining the crystal phase of the HAp granules, the crystallinity was assessed by measuring the full width at half maximum (FWHM) using a peak with a $2\theta=26°$ (002) diffraction. The crystal size D_{002} was also estimated as follows using the Scherrer's equation[13].

$D_{002}=0.9\lambda/\beta\cos\theta$

where λ is the wavelength of Cu-Ka1, β is the FWHM, and θ is the Bragg angle of the diffraction pattern (002).

Surface observation

The surface morphology of the HAp granules was observed under a scanning electron microscope (SEM, JSM-6340F, JEOL, Japan). The HAp granules were coated with Au-Pd alloy. The accelerating voltage was set at 15.0 kV.

Specific surface area

The specific surface area (SSA) of the HAp granules was measured by the BET method based on adsorption isotherms of nitrogen gas using a surface area measuring instrument (GEMINI2370, Shimadzu, Japan). The SSA was calculated by the quantity of gas adsorbed onto the 1 g sample and expressed as square meters per gram (m^2/g).

Calcium dissolution from HAp granules

100 mg of HAp granules were immersed in 10 ml distilled water (pH 6.4) during 24 or 72 h at 37°C. After the granules were filtered with a Millipore filter having a pore size of 0.2 μm, the

amount of calcium dissolved from HAp granules was measured by an inductively coupled plasma atomic emission spectrometer (ICP spectrometer, Vista-MPX, SII, Japan).

Bps Release from Bps-HAp Composites

A total of 10 mg each of the Bps-HAp composites prepared in section "Bps-HAp composites" was immersed in 1 ml of distilled water at 37°C for 24 or 72 h. After the composites were filtered using a Millipore filter with a pore size of 0.2 μm, the concentration of Bps in the residual solution was measured using a liquid chromatography mass spectrometer (LC/3DQMS, M-8000, Hitachi, Japan).

Cellular Effects of Bps Released from Bps-HAp Composites

Osteoclasts were prepared from a rabbit (7-day-old male Japan white rabbit) according to a method previously described [14]. Briefly, the tibiae and femur were divested of muscle and periosteum, cut across at their epiphyses, and then split longitudinally with a razor blade in medium 199 (Gibco) containing 10% fetal bovine serum, penicillin G (100 μg/mL), streptomycin (100 μg/mL), and amphotericin (0.25 μg/mL) antibiotics. Cell suspensions containing osteoclasts were obtained from inside the bony shafts by scraping out the trabecular bone, suspending the bone particles in the medium, and releasing the cells by pipetting. The osteoclast-rich suspension was centrifuged at 300 × g for 5 min at 4°C, and the osteoclast-rich fraction was collected. In each well of a 96-well plate with tilted square wells (HTS BD Falcon, UK), a 5.5-mm diameter bone disk was placed at the bottom, and 200 μl of culture medium containing the osteoclasts was added. MEM containing 10% FBS and antibiotics was used as the culture medium. The concentration of the collected fraction was 0.8×10^6 cells/ml. The cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2.

An osteoclast culture experiment was conducted by establishing the following three groups: negative control group (nothing administered); HAp granule group; and Bps-HAp

composite group. As shown in Figure 1, using a 96-well multi-well insert (BD Falcon, UK), 2.0 mg of either HAp granules or Bps-HAp composites were positioned on a filter with a pore size of 1.0 μm so that the composites or granules did not come into direct contact with the osteoclasts placed at the bottoms of the wells. The cells were then incubated at 37°C for 72 h in a humidified atmosphere of 5% CO_2.

After incubation, the osteoclasts were fixed for 5 min using a PBS (-) solution containing 2.5% glutaraldehyde (Wako, Japan) and then stained using a tartrate-resistant acid phosphatase (TRAP) stain (Hokudo, Hokkaido, Japan). The number of osteoclasts was counted on the bone disk under an optical microscope. In the present study, the osteoclasts were defined as TRAP-positive cells with more than 3 nuclei. The survival rates of the osteoclasts on the bone disks in either the HAp or Bps-HAp composite groups were calculated with respect to the mean number of osteoclasts on the bone disk in the control group.

All experiments were performed in accordance with the guidelines for experimental laboratory animals in the animal facility of Tokyo Dental College.

Statistical Analysis

All measurements were conducted in triplicate. The data were analyzed using an analysis of variance (two-way ANOVA, factor A: sintering temperature, factor B: immersion time) followed by Fisher's PLSD (protected least significant difference) method for multiple comparisons between pairs.

RESULTS

Characterization of HAp Granules

The X-ray diffraction patterns for the HAp granules were shown in Figure 2, All patterns were assigned to hydroxyapatite (JCPDS card 9-432). The full width at half maximum (FWHM) of the $2\theta=26°$ (002) diffraction was 0.424 for HAp400, 0.376 for HAp800, and 0.329 for HAp1200. The FWHM values were smaller with a higher sintering temperature. The crystal

size was 22.3nm for HAp400, 25.2nm for HAp800, and 28.8nm for HAp1200.

Figure 3 shows SEM images of HAp granules sintered at the different temperatures. Higher sintering temperatures were associated with denser surfaces and larger grain size.

The specific surface area (SSA) for HAp400 and HAp800 was 46.2 m^2/g and 17.9 m^2/g, respectively. The SSA for HAp1200 was below the measurement limit (=0.1 m^2/g). The SSA decreased with an increase in the sintering temperature.

Figure 4 shows the concentration of dissolved calcium from each HAp granule after 24 or 72 h immersion. After 24 h immersion, the concentration of dissolved calcium from HAp400, HAp800, and HAp1200 was 2.20 ±0.06 ppm, 1.38 ±0.18 ppm, and 0.08 ±0.01 ppm, respectively. The concentration of dissolved calcium from the HAp granules decreased with an increase in the sintering temperature, and significant differences in the dissolved calcium existed among the HAp granules ($p<0.01$). After 72 h immersion, the concentration of dissolved calcium from HAp400, HAp800, and HAp1200 was 3.02 ±0.11 ppm, 3.06 ±0.53 ppm, and 0.22 ±0.10 ppm, respectively. No significant differences were noted between HAp400 and HAp800 ($p>0.05$). At 400 and 800°C of the sintering temperature, the concentration of dissolved calcium from the HAp granules increased with immersion time ($p<0.01$).

Concentration of Bps Released from Bps-HAp Composites

Figure 5 shows the concentration of Bps released from each Bps-HAp composite after 24 or 72 h immersion. After 24 h immersion, the concentration of Bps released from Bps400 was 0.12 ±0.04 mM, from Bps800 was 0.05 ±0.01 mM, and from Bps1200 was 0.03 ±0.01 mM. After 72 h immersion, the concentration of Bps released from Bps400 was 0.30 ±0.06 mM, from Bps800 was 0.17 ±0.02 mM, and from Bps1200 was 0.10 ±0.02 mM. A significant difference in the Bps released between the Bps400 and the Bps800 or the Bps1200 existed after 24 or 72 h immersion ($p<0.01$). Bps release depended on the sintering temperature of the HAp and the immersion period.

Osteoclast Survival Rate

Osteoclasts were defined as TRAP-positive cells with more than 3 nuclei (Figure 6). Figure 7 shows the osteoclast survival rate for HAp granules and Bps-HAp composite groups after 72 h incubation. With the HAp granules, the osteoclast survival rate was 98.3 ±4.6% in HAp400, 97.8 ±4.6% in HAp800, and 99.0 ±7.0% in HAp1200. The differences in the survival rates among the groups were not significant ($p>0.05$). With the Bps-HAp composites, the osteoclast survival rate was 36.1 ±18.2% in Bps400, 58.8 ±20.7% in Bps800, and 96.2 ±9.9% in Bps1200. Compared to that of the control, osteoclast survival decreased significantly for Bps400 and Bps800 ($p<0.01$).

DISCUSSION

The objective of the present study was to establish a local administration method for Bps, which is a bone resorption inhibitor. As the technique for local administration, a DDS system using prepared HAp with carriers of Bps was investigated. We then examined the correlation between the characteristics of HAp and the released Bps and the effect of the released Bps on the osteoclasts.

SEM observation indicated that the HAp particles were larger with higher sintering temperatures. Compared to that of HAp400, the surface of HAp1200 was smoother and more even. This agrees with the results for SSAs, with higher sintering temperatures being associated with smaller SSAs. These findings have been documented by Kamoi et al.[12], Ducheyne et al.[15], Saalfeld et al.[16], and Oiso et al.[17]. Many studies have found that the crystallinity of HAp is affected by sintering temperature. Kamoi et al. investigated the relationship between sintering temperature and crystallinity, and reported that a higher degree of crystallinity (sharper peak given by X-ray diffraction) was obtained with a higher sintering temperature [12]. The effects of varying sintering temperature are well documented and some discussion on nucleation

temperature is definitely warranted. In general, below the nucleation temperature (around 750-800) the HA has smaller crystal size, more porosity, and therefore can be expected to have faster dissolution. This was confirmed by this study. In the present study, the crystallinity was the lowest for HAp400 and the highest for HAp1200. These findings suggest that altering the sintering temperature may change not only the surface morphology of HAp but also the specific surface area (SSA) and the crystallinity. In other words, altering the sintering temperature can control the physical properties of HAp.

It is important to investigate the solubility of HAp for a DDS system with Bps-HAp composites because Bps have a high affinity for calcium in the HAp. The present results showed that the solubility was the highest in HAp400 and the lowest in HAp1200, suggesting a lesser degree of calcium dissolution with a higher sintering temperature. In general, materials with large SSAs have larger areas of contact with solvent, resulting in higher solubility. HAp granules formed at a low sintering temperature have irregular surface structures and large SSAs and, therefore, the solubility is higher. In addition, the results of crystallinity analyses by X-ray diffraction showed that when the sintering temperature was low, crystallinity was low, but HAp solubility was high. Kamoi et al. investigated the relationship between crystallinity and solubility and reported that lower sintering temperatures were associated with lower crystallinity and higher solubility [12]. The present results support their findings. Consequently, the regulation of HAp solubility appears to be possible by adjusting the sintering temperature to alter surface morphology, SSA, and crystallinity. Thus, altering the sintering temperature is considered a useful method for controlling HAp solubility. The concentration of Bps released from the Bps-HAp composites was related to the concentration of calcium dissolved from the HAp. Bps release was highest for Bps400, but was lower for composites with higher sintering temperatures. Therefore, controlling HAp solubility regulates the concentration of Bps released from the composites.

The relationship between calcium dissolution and Bps release was compared between

Bps400 and Bps800. The concentration of calcium dissolution after 24 and 72 h immersion for Bps400 was 1.6 and 1.0 times higher, respectively, while the concentration of released Bps was 2.4 and 1.8 times higher, respectively. The concentration of released Bps was thus greater than that of calcium dissolution. If a linear correlation between calcium dissolution and Bps release could be confirmed, then the concentration of released Bps for Bps400 should be 1-1.6 times greater than that for Bps800. However, the concentration of released Bps for Bps400 was 2.4 times higher, suggesting that released Bps is dependent on factors other than calcium dissolution. Note that for the SSA value of HAp, the value of Bps400 was 2.6 times higher than that of Bps800, suggesting a close correlation between SSA and released Bps. As mentioned above, a lower sintering temperature of HAp as a Bps carrier is associated with a greater SSA. This suggests that since Bps adsorbs on the surface of HAp, more Bps adsorbs onto HAp prepared at a lower sintering temperature. The higher concentration of Bps released from Bps-HAp composites is considered higher due to the large amount of Bps adsorption. Figure 8 summarized the correlation between a) solubility and crystallinity of HAp, b) solubility and SSA of HAp, c) concentration of Bps release and crystallinity of HAp, and d) concentration of Bps release and SSA of HAp. These graphs showed that both crystallinity and surface area of HAp influence not only solubility of HAp but also the Bps release. On the amounts of Bps adsorption, however, further study is necessary to determine the suitable soaking time in Bps solution because the both adsorption and releasing reaction of Bps is occurred simultaneously, indicating that the amounts of Bps adsorption can be depended on the soaking time.

The above findings explained that the concentration of Bps released from the Bps-HAp composites could be controlled by the solubility of HAp and the adsorption amounts of Bps by altering the sintering temperature of HAp. There were significant differences in the osteoclast survival rate between the control and that of any of the HAp groups, while the rate of dissolution of calcium from the HAp granules was significantly ($p<0.01$) different between HAp1200 and HAp400, or HAp800. This result suggests that the dissolution of calcium from

any of the HAp granule groups had no effect on the osteoclast survival rate.

Bps400 showed the lowest osteoclast survival rate. No significant differences in osteoclast suppression between Bps1200 and the control were seen. A lower HAp sintering temperature was associated with higher Bps release. This result indicates that the degree of osteoclast suppression is dependent on Bps release. Consequently, Bps released from Bps-HAp composites reduces the osteoclast survival rate, suggesting that Bps-HAp composites are applicable to the bone-deficient areas as a drug delivery system.

Hughes et al. reported that the apoptotic osteoclast rate was approximately 20% in a direct dose of pamidronate at a concentration of 10^{-5} M in vitro [18]. This Bps concentration was the same as that of the released Bps from Bps1200 in this study; however, the osteoclast survival rate was the same as the control. Taken together, in terms of apoptosis, we concluded that Bps1200 is not able to inhibit osteoclasts; Bps400 is the most effective for osteoclast inhibition. A discrepancy of concentration for the osteoclast survival rate between reported study[17] and the results of this study may be due to the difference in the administration method (direct in the reported study, indirect in this study) as well as the cells used.

Bisphosphonates have been reported not only as potent inhibitors of osteoclastic bone resorption, but also have a direct effect on osteoblasts [3, 19-21]. Bisphosphonates are very potent inhibitors of bone resorption. The mechanism of this phenomenon is still unclear but it is probably at the cellular level, especially as it applies to the activity of osteoclasts. Tyrosine phosphatase activity plays an important role in osteoclast formation and function, and is a putative molecular target of bisphosphonate action [22]. It is also reported that while pamidronate does not inhibit the gene expression of the putative tooth eruption molecules, it does increase osteoclast size [23]. Gandolfi et al. demonstrated that good differentiation and osteoblastic activity occur in cells that are in contact with bisphosphonates [24]. Giuliani et al. suggested that bisphosphonates might have, in vivo, a potentially relevant influence on cells of osteoblastic lineage distinct from their inhibitory action on osteoclasts [25]. Although further investigations

are necessary to clarify the effects of Bps released from Bps-HAp composites on bone regeneration, the use of Bps-HAp composites could become advantageous.

In this experiment, we measured the concentration of calcium released from HAp. However, the concentration of calcium released from the Bps-HAp composite was not estimated. Bps chemically binds to calcium so that the concentration of released calcium varies from that of the original HAp.

In addition, long-term Bps release must be examined to ascertain whether the release is continuous over longer periods. More detailed studies are necessary to clarify these items.

In summary, the present study revealed that 1) HAp solubility depends on the sintering temperature; 2) The concentration of released Bps could be controlled by regulating the sintering temperature of HAp as a carrier; and 3) Bps released from Bps-HAp composites reduced the number of osteoclasts with reducing the sintering temperature of HAp. These findings indicate that Bps-HAp composites could be locally administered as a drug delivery system to areas with bone resorption such as cancer-induced osteolysis, wound after extraction of tooth as well as bone bed around the implants.

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Legend for figures

Figure 1 Illustration of experiment for osteoclast survival rate.

Figure 2 X-ray diffraction patterns for the HAp granules.

Figure 3 SEM images of HAp granules: (a) HAp400, (b) HAp800 and (c) HAp1200.

Figure 4 Concentration of dissolved calcium from HAp granules. Identical letters indicate no significant difference ($p>0.05$).

Figure 5 Concentration of Bps released from HAp-Bps composites. Identical letters indicate no significant difference ($p>0.05$).

Figure 6 Osteoclasts (arrow) stained by TRAP.

Figure 7 Osteoclast survival rate. Identical letters indicate no significant difference ($p>0.05$).

Figure 8 Correlation between a) solubility and crystallinity of HAp, b) solubility and SSA of HAp, c) concentration of Bps release and crystallinity of HAp, and d) concentration of Bps release and SSA of HAp.

Figure 1

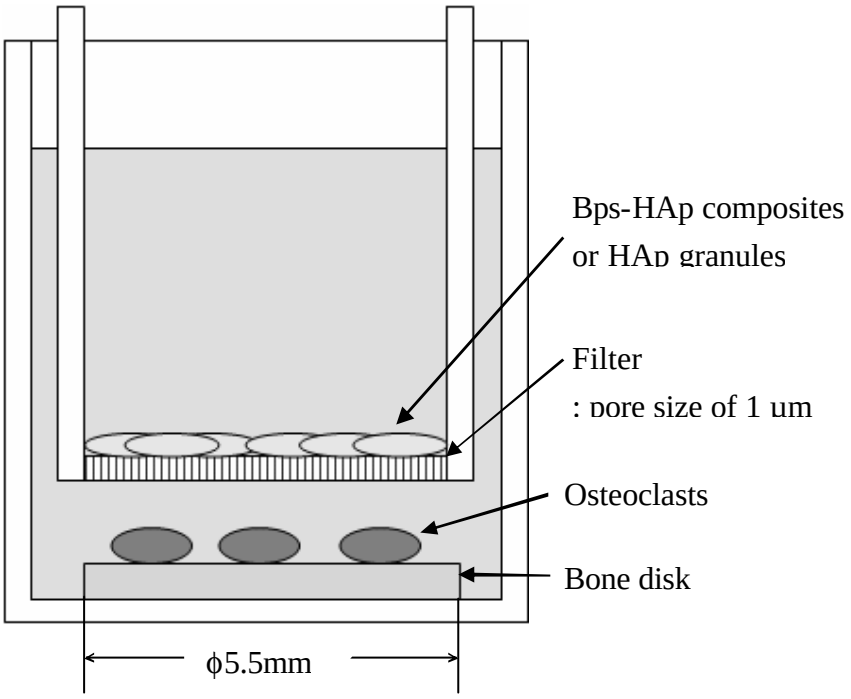

Figure 2

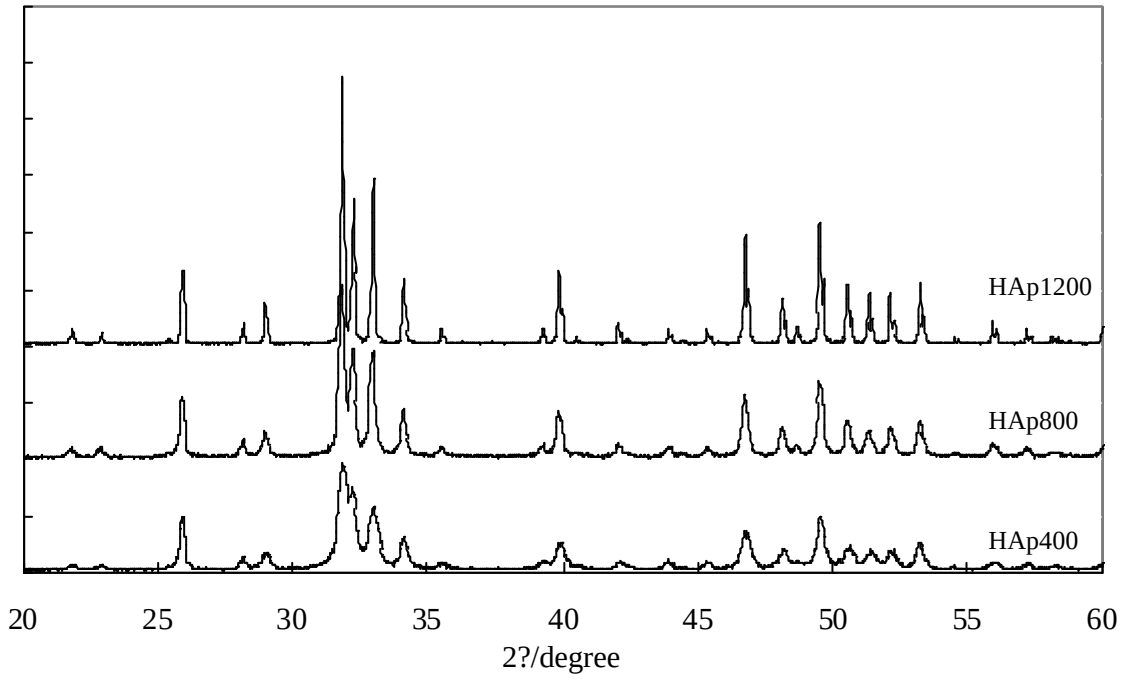

Figure 3

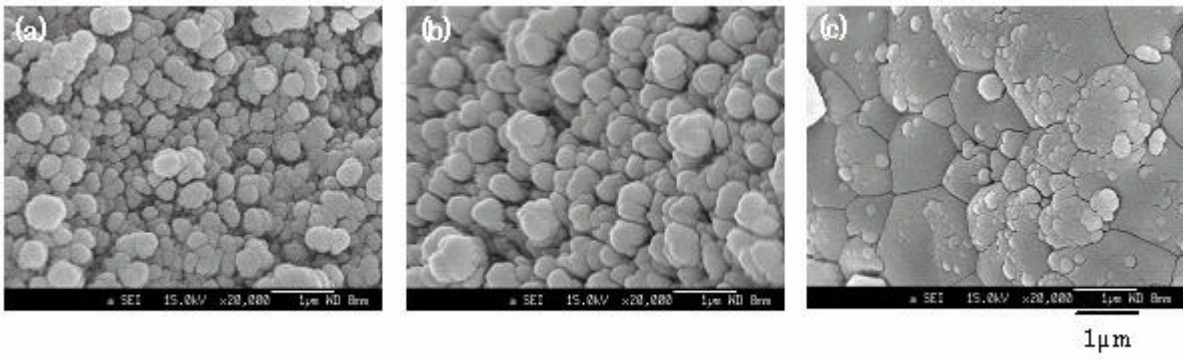

Figure 4

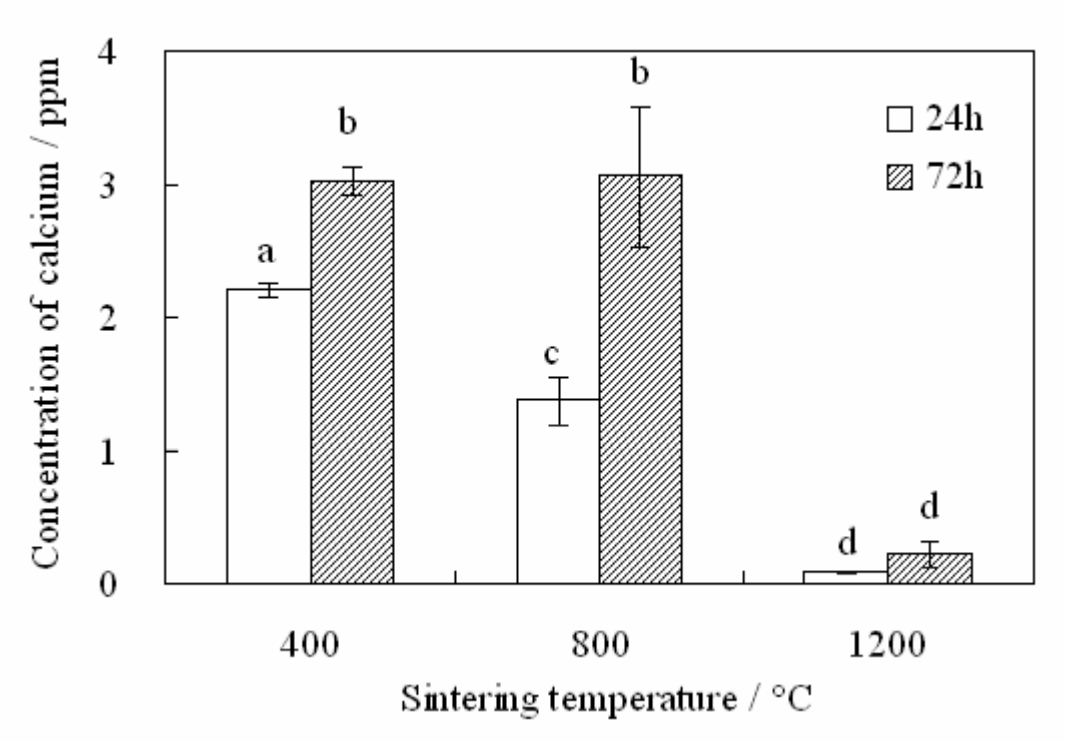

Figure 5

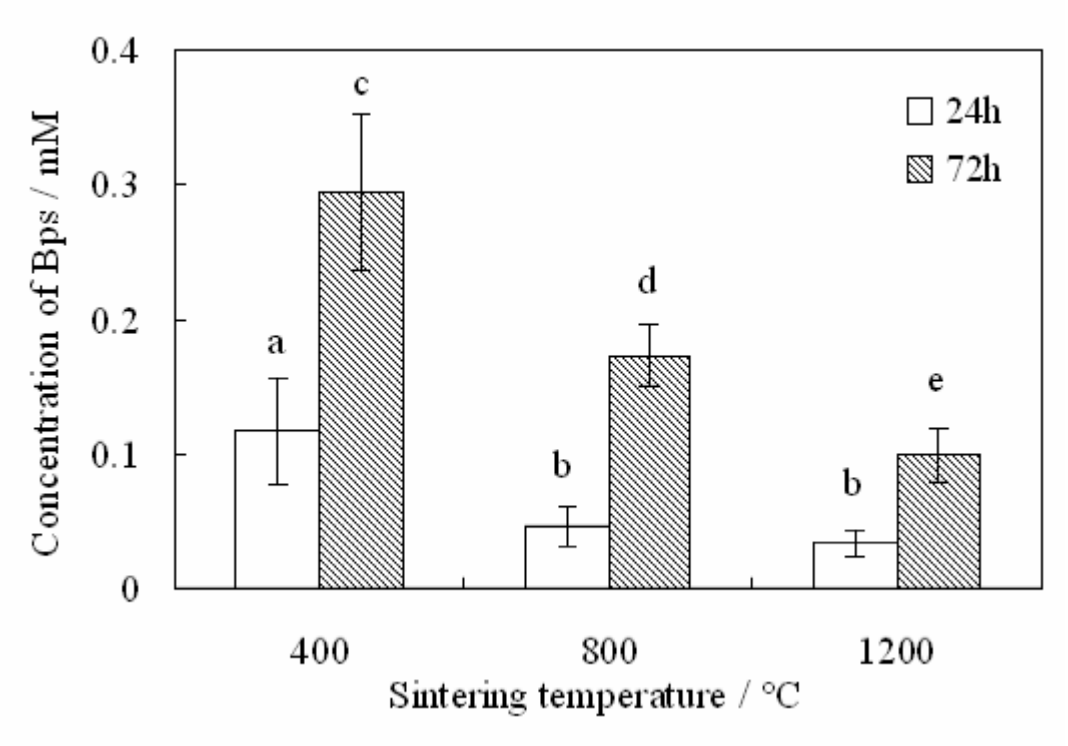

Figure 6

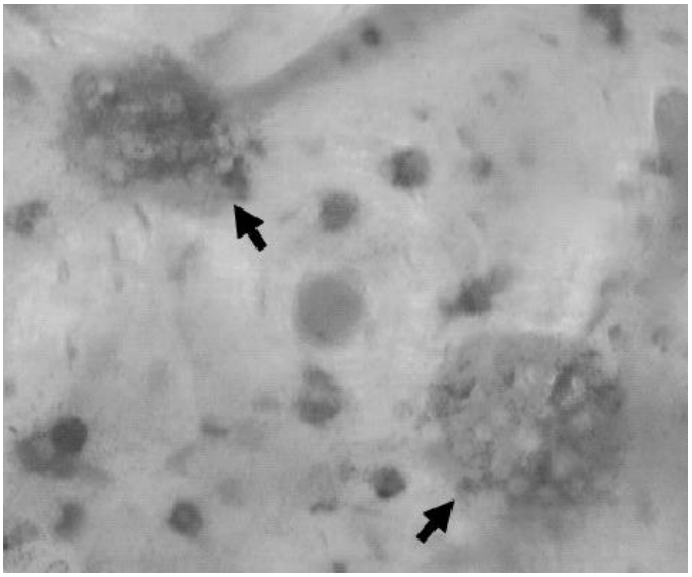

Figure 7

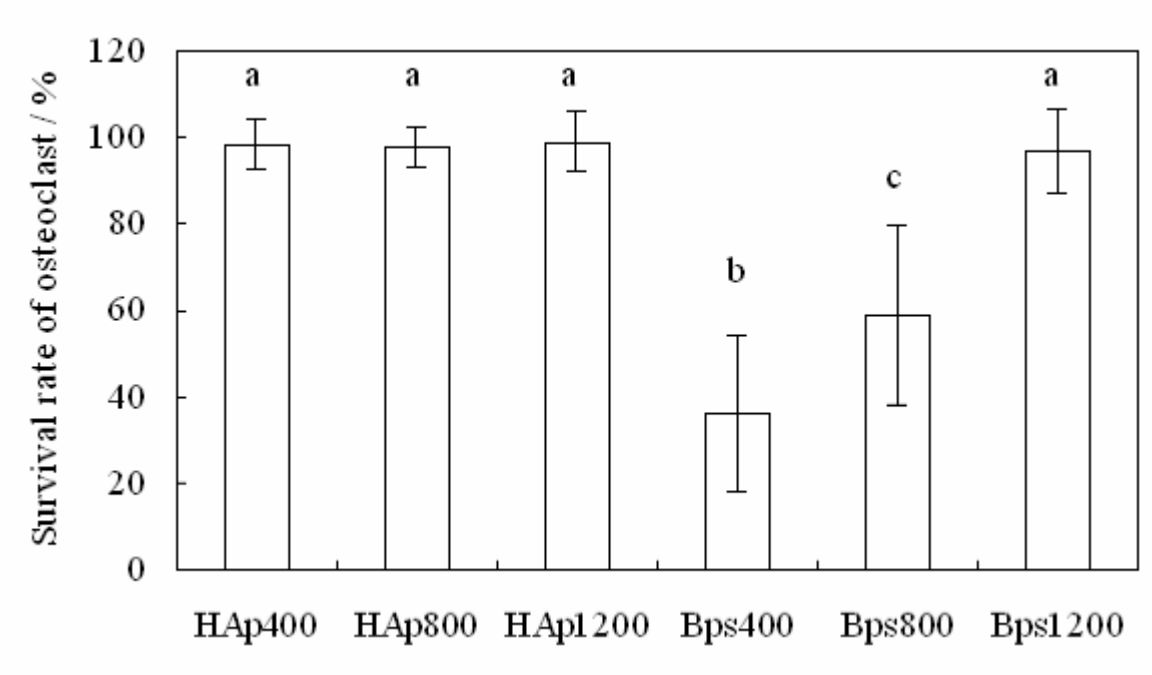

Figure 8