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Drug-loaded porous spherical hydroxyapatite granules for bone regeneration

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Abstract Porous spherical hydroxyapatite (HAp) granules, which are not only can be used for bone void filler, but also drug delivery systems, were prepared using a liquid nitrogen method. Various pore and channel structures of spherical granules were obtained by adjusting the ratio of water to HAp powder and the amount of sodium chloride (NaCl). By using the water to powder ratio at 2.0 ml/g and the amount of NaCl at 15 wt% by powder, the spherical granules have optimal pore volume, micro-channel structure and strength to handle as well as the ability to work as a drug delivery system. When the NaCl content was 15 wt%, the micro-channel structure was changed, but the pore volume was maintained. For the drug release test, dexamathasone (Dex) was loaded as a model drug on the prepared HAp granules by the immersion method, and the drug release behavior was curved by a UV/vis spectrophotometer. As a result, different drug release behavior was observed according to micro-channel structural differences. Therefore, it was concluded that the NACl could be applied as the pore and micro-channel structure control agent. Porous spherical HAp granules, which were fabricated by a liquid nitrogen method, show potential as bone void filler with the ability of controlled drug release.

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1 Introduction

Porous calcium phosphates are gaining clinical acceptance as synthetic bone graft substitutes or graft extenders, where the supplies of autograft and allograft is not adequate for clinical demand. They are produced via a number of routes such as ceramic slip foaming [1], positive replication of reticulated foam scaffolds [2], burnout of sacrificial porogens such as polymer beads [3], and techniques that exploit naturally occurring porous calcium-based structures such as the hydrothermal conversion of either coral [4] or bone [5]. The philosophy behind the idea of using the porous structures in bone repair stems is from the demonstration of thinner fibrous encapsulation around porous implants that result in mechanical interlocking during the tissue penetration [6, 7]. Furthermore, an open macroporous structure similar to that of cancellous bone will promote the complete infiltration of bone tissue, bone marrow, and blood vessels, as occurs when autografts and allografts are used [8].

Since calcium phosphate-based bioceramics such as hydroxyapatite [$(Ca_{10}(PO_4)_6(OH)_2)$, HAp] are known for their excellent biocompatibility due to their similarity in composition to the apatite found in natural bone [9]. HAp has been frequently used for the fabrication of highly porous, interconnected scaffolds and isotropicized pore structure in the last decade [10–12]. Various types of HAp bone grafts such as dense and porous blocks, dense and porous granules, and powder form [13] have been

clinically and experimentally used. However, it is important to make porous matrices that enable cell migration through the pores, as well as providing suitable conditions for nutrient transport, tissue infiltration, and vascularization [14, 15]. Furthermore, it is known that spherical-shaped bone grafts are as suitable for implantation as injectable bone cements [16, 17]. When spherical granules are uniformly packed, they can contribute to cell migration and extracellular matrix (ECM) growth through the vacancies that were formed between spherical granules (Fig. 1a) [16].

Previous research from other groups focused on anti-inflammatory or anti-bacterial drug release from HAp, as it is common that implants could become inflamed, causing a local defect after surgery [18]. Therefore, such a drug was loaded into the porous HAp spherical granules, which were fabricated by liquid nitrogen and freeze drying method.

The porosity, pore size and micro-channel were varied with the additives such as water, binder and reagents. The pore volume of the HAp granules were also altered by changing water content and forming different pore and micro-channel structures using NaCl. It was thought that NaCl as a sintering additive could change the pore and micro-channel structure in the HAp granules after sintering. We hypothesized that the drug release rate can be controlled by complex micro-channel structures of the HAp granules (Fig. 1b). Dexamethasone (Dex) is used as a model drug, which acts as an anti-inflammatory and has many benefits. Briefly, Dex induces osteoblastic differentiation in vitro, and increases alkaline phosphotase activity, expression of osteocalcin, and bone sialoprotein [19–22].

This study presents the preparation process of the porous spherical HAp granules with a novel method for fabrication of bone void filler, creating various pore and micro-channel structures controlled by the addition of water and NaCl, and release behavior of Dex from the porous HAp granules.

2 Materials and methods

2.1 Preparation of porous spherical HAp granules

Porous spherical HAp granules were fabricated using a liquid nitrogen and freeze drying method. Commercial HAp powder (OssGen Co., Daegu, Korea) was used for the preparation of the distilled water-based ceramic slurry. Binders (3% high molecular weight polyvinyl alcohol, 3% carboxymethylcellulose, and 5% ammonium polyacrylate dispersant) were added to the slurry mixture to improve sintering and the stability of the scaffold structure. The HAp slurry for granule fabrication was prepared by gradually increasing H_2O/HAp ratio 1.5, 2 and 4, and gradually increasing the NaCl content of 3, 15 and 30 wt% by powder to change the pore and micro-channel structure of the spherical granules. The group codes that were assigned to different composition slurry are provided in Table 1. The slurry was stirred in several steps at a low rotation speed of 1,000 rpm. The temperature of the slurry was adjusted to room temperature during the low rotation speed stirring. When the slurry mixture was homogenous, the rotation speed was increased to 5,000 rpm and the slurry

Table 1 HAp slurry component of each group. Group Nx is what was added NaCl in group H2 component

	wt% by HAp	H_2O/HAp ratio (ml/g)		
		1.5	2	4
Polyvinyl alcohol	2	Group H1	Group H2	Group H3
Carboxymethyl cellulose	1			
Ammonium polyacrylate	5			
Group H2	NaCl wt% by HAp			
	3	15	30	
	Group N1	Group N2	Group N3	

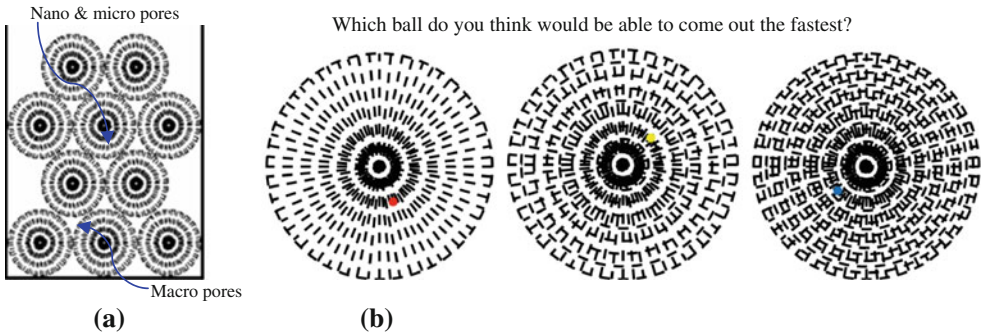

Fig. 1 **a** The scheme of bone defect which is filled by porous HAp spherical granules. The filled defect has various sizes interconnective pores. **b** The schematic representation of our hypothesis. Nobody would deny the fact that the answer to the question is the red ball

was stirred for 10 h at this rotation speed. The temperature of the slurry was also reduced to 15°C during high-speed stirring. The slurry was dropped into liquid nitrogen using a 24G needle (inner diameter of 0.47 mm) syringe with drop/second where spherical-shaped granules instantaneously formed. The spherical granules were lyophilized for 24 h and then heated to 1,230°C by increasing the temperature at 5°C/min. The sintering time at this temperature was set to 5 h. The sintered granules were then cooled back to room temperature at cooling rate of 5°C/min (Thermo Scientific, Asheville, USA).

2.2 Material characterization

Scanning electron microscope (SEM, EVO 40, ZEISS, Peabody, USA) analysis was performed to compare the surface morphology of each group and the cross-section of pore distribution. The element analysis was conducted by energy dispersed spectroscopy (EDS, HORIBA, Kyoto, Japan) to identify the composition of the residue. The phase composition of the HAp granule after thermal treatment was determined by XRD analysis with the use of an X-ray diffractometer (Rigaku D/Max, Tokyo, Japan) on groups H2, N1, and N2. Cu Kα radiation was used at the opening condition of 40 kV and 20 mA. The XRD data were collected over the 2-theta range of 20–40°, with a step size of 0.02°. Identification of the phases was achieved by comparing the diffraction patterns of the composites with the standard JCPDS cards.

To estimate the pore volume of the HAp granules and compare them with the morphology on the SEM images, dried HAp granules were weighed and immersed in distilled water and ethanol. The liquids were allowed to penetrate the HAp granules for a day under the vacuum. The HAp granules were then removed from the liquid and gently wiped and reweighed. The pore volume in the HAp granules was determined using the formula created by Paul and Sharma [13].

2.3 Drug loading and release studies into HAp granules

The drug release test was performed to verify the different release rates according to structural differences. The HAp granules were immersed in Dex (Sigma-Aldrich, St. Louis, MO, USA) solution (0.01 g/ml) under the vacuum at 20°C and allowed to stand for 24 h. These HAp granules were lyophilized for 24 h, and the release of Dex from the HAp granules was measured by placing the samples in a 3 ml vial and immersing them in 2 ml of phosphate-buffered saline (PBS, pH 7.4) at 37°C for up to 14 days under static condition. The PBS solution was collected and replaced with fresh PBS at predetermined time intervals. The amount of the released Dex was calculated using a UV-vis spectrophotometer (UVD-3200, Labomed, Culver, CA, USA) at 242 nm [23].

3 Results and discussion

3.1 Granule morphology and size

The method described here seems to be suitable for the preparation of porous spherical HAp granules ranging from 500 to 1,000 μm in diameter. However, if different needle gages were used and different dropping speeds were applied, a varying size of granules would be formed. Granules were prepared by varying the water and NaCl content. Different commercial grades of HAp granules are available, which are used for a wide range of applications such as general dental, periodontal, and oral/maxillofacial surgical procedures, including augmentation, fill-in and repair. Some examples are INTERPORE200 granules (425–1,000 μm diameter), PRO OSTEON500 granules (1–9 mm), Osteograf/N (225–400 μm), OSTEOGEN (300–1,000 μm) and BIO-OSS. Granules with an average overall diameter of 425–600 μm are recommended for periodontal applications and with an overall diameter of 425–1,000 μm for oral surgical applications [24], and 300–400 μm for alveolar ridge augmentation. The prepared HAp granules had an overall spherical geometry, as evidenced by Figs. 2 and 3. Before heat treatment, an individual HAp granule has a smooth surface texture surrounded by polymer binder. After heat treatment, the polymers were burned out and HAp crystals were visible. The surface of the granule was observed to have pores of 2–15 μm distributed over its entire surface. It is known that surface texture [25], and implant shape [25, 26] affect tissue response. Smooth and round particles induce a less favorable reaction than rough and angular materials. In addition, it has been reported that significantly faster ingrowth of bone was observed with rounded granules than

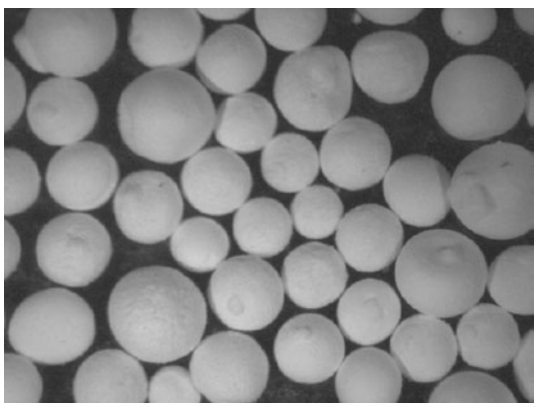

Fig. 2 Optical micrograph of spherical HAp granules (400–700 μm diameters)

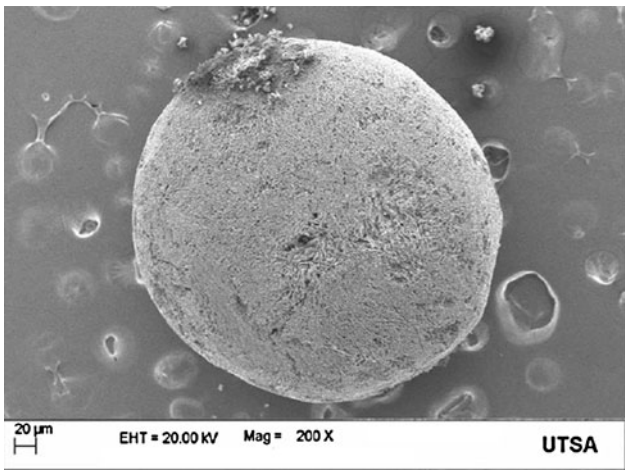

Fig. 3 SEM micrograph of spherical HAp granule

polygonal-shaped granular material [27], and rounded particles seem to be clinically more suitable for implantation in the oral cavity [26], especially in stress-bearing areas such as the mandibular alveolus. Spherical particles were preferred because of their unique packing characteristics. Spheres, when packed together, form a matrix with uniform pore distribution between particles, and this configuration promotes efficient conduction between bone particles [28].

3.2 Pore and micro-channel structure change

The SEM images of groups Hx and Nx are shown in Figs. 4 and 5. As shown in the images, the spherical HAp granules had unique structures with pore and micro-channels continuing from the center to outside in a radial shape. The micro-channels were larger with increased water content (Group H1 to Group H3). Such structure shape was believed to be formed by the growth of ice crystals and lyophilization. The frozen HAp granules were sublimed by lyophilization, and the structures retained their shape after sintering. As shown in Fig. 4, there was debris on the surface of group H3, which did not have appropriate strength for handling. Group Nx (Fig. 5) images presented different aspects compared to group Hx. Group N1 is shown to be similar to group Hx. However, the pore and micro-channel of groups N2 and N3 were irregular with the increasing content of NaCl. When the frozen HAp granules were placed in the vacuum freeze dryer, the NaCl remained in the water path. This formed the radial shape of the micro-channels in group Hx. The remaining NaCl is believed to have played a role as a grain growth accelerator during the sintering process. Therefore, the grains grew and the pores shrank with the increasing NaCl content. Finally, the sintered HAp granules were densified when the NaCl was burned out from the pore and micro-channels.

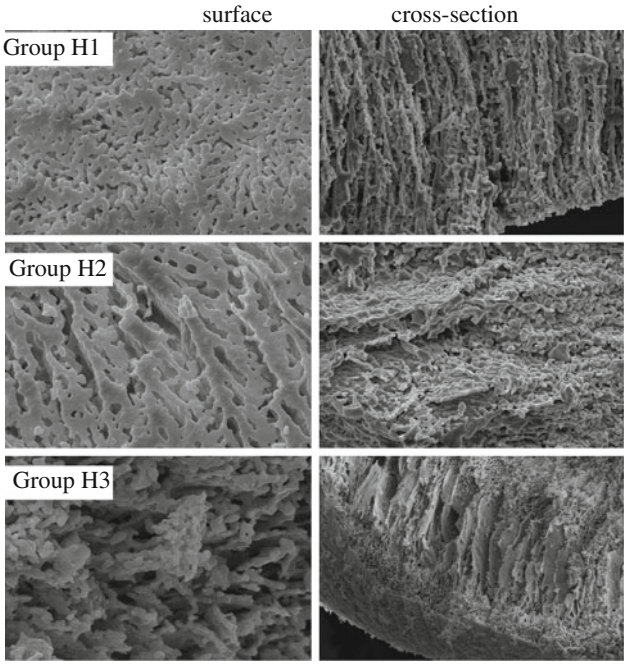

Fig. 4 SEM images for each group show various sizes of pores and pore channels. As increasing water content of HAp slurry, the micro-pores were revealed certainly. (Magnification of surface images is ×5,000, and that of cross-section images is ×1,000)

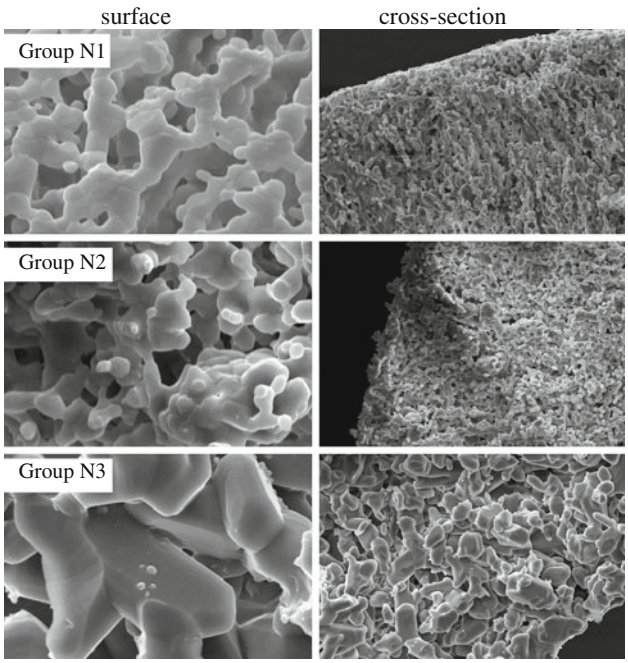

Fig. 5 SEM images for each group show various sizes of pores and pore channels. As increasing the content of NaCl, the internal pore structure changed more densely. (Magnification of surface images is ×5,000, and that of cross-section images is ×1,000)

As shown in Fig. 6, the pore volume showed the same pattern as the results of the SEM analysis. The pore volume also increased with increasing water content of the HAp

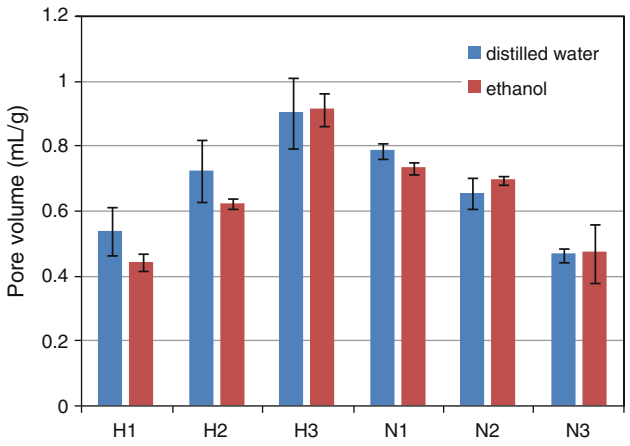

Fig. 6 The results show the pore volume of each group

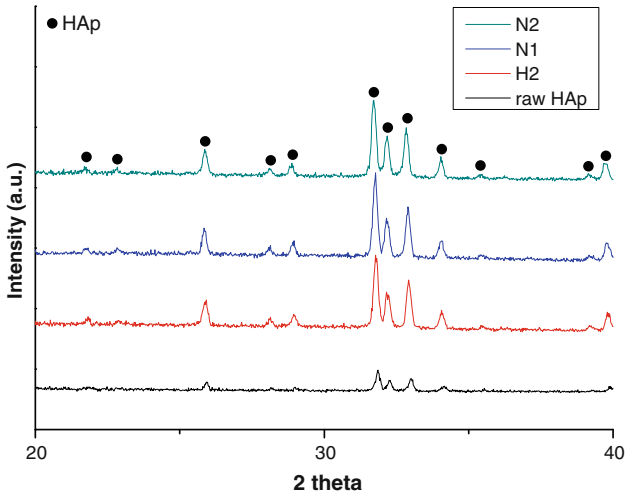

Fig. 7 The results of XRD show that the polymer and NaCl burned out and the only HAp peaks appeared

slurry and decreased with increasing NaCl content of the slurry. The results show similar results as the experiments performed with water and ethanol.

3.3 XRD and EDS studies

X-ray diffraction analysis indicated that the particles did not contain any distinguishable crystalline impurity, and the pattern shown in Fig. 7 matched the standard pattern for hydroxyapatite (JCPDS file number 9-432). It was confirmed that the polymer binders and NaCl burned out without any residues, and crystallization occurred after sintering, as revealed by the results of XRD and EDS (Figs. 7 and 8). The EDS study (Fig. 8) clearly reveals the presence of Na^+ and Cl^- at the green body of the HAp granule before sintering. On the other hand, there is no evidence of Na^+ and Cl^- at the sintered HAp granule.

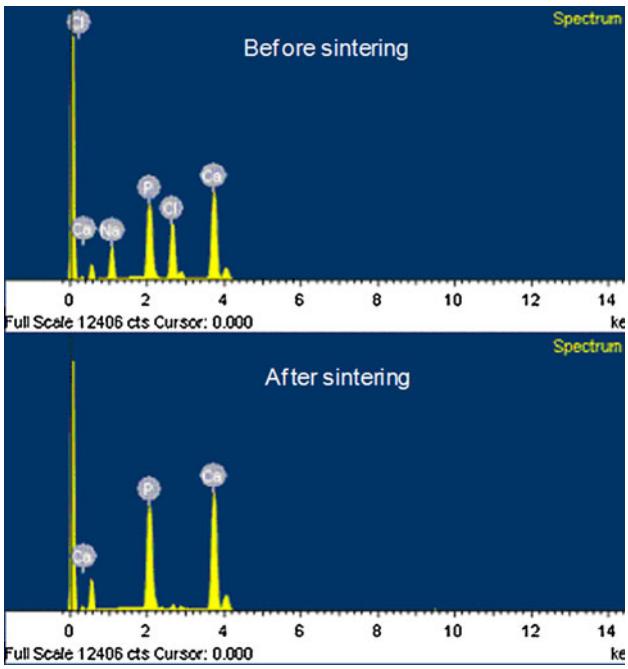

Fig. 8 The results of EDS show that the polymer and NaCl burned out and the only calcium and phosphorus peaks appeared

3.4 In vitro Dex release

Porous HAp granules can be utilized for the delivery of macromolecules, protein drugs or polypeptides. Protein drugs, when encapsulated, may denature within the polymer matrix [29], causing a loss of biological activity and possible changes in immunogenicity. This has been caused mainly by the interaction between the drug and the polymer or the solvent used, as well as the temperature involved. In addition, the degraded byproducts may cause side effects, thus reducing the intended effect of the drug. However, hydroxyapatite is a biocompatible material that could be used as a matrix for purification of the proteins themselves, which does not seem to cause any side effects and is shown to be non-toxic [30, 31] and non-carcinogenic [30]. Thus, the HA system could be effectively used as a sustained delivery device in humans.

The drug release test was performed with groups H2, N1, and N2. Group H2 had appropriate strength for handling, unlike group H3, and the pore volume of group H1 was too low compared to group H2. The pore volume of group N3 was too low and that of group N1 and N2 was similar to group H2. However, the drug release test plan was set up to compare the release rate accordance with internal pore structure. Therefore, groups H1, H3, and N3 were excluded from the test. Figure 9 shows the results of the drug release behavior test. Groups H2, N1, and N2 were similar in terms of loaded drug content. However, as shown in Fig. 9, the drug release behavior of group H2, N1, and

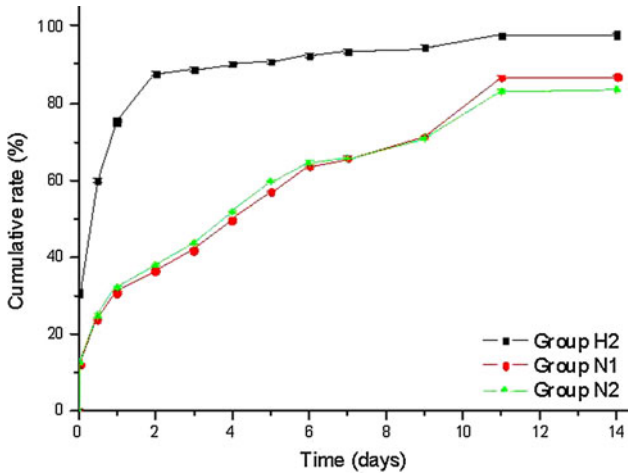

Fig. 9 The drug release behavior of group H2, N1, and N2. The behavior was showed different pattern as different internal pore structures

N2 presented with different patterns. The Dex was released over a period of 14 days in group N1 and N2, whereas group H2 was shown to burst release Dex within 2 days. Hence, it was thought that the irregular pore and micro-channel structure of groups N1 and N2 affected the delay of the drug release.

4 Conclusion

A new method for the preparation of porous spherical HAp granules has been developed using the liquid nitrogen method. Since smooth granules cause less inflammatory response and facilitate faster bone ingrowth, compared to irregular granules, the porous spherical granules prepared appear to be a suitable implant material in many orthopedic and maxillofacial applications as fillers or packing material. Although it has been shown that the granules are pure HAp, their biological performance needs to be evaluated in a suitable animal model.

The major limiting factor in utilizing protein drugs for sustained delivery is the lack of suitable delivery systems. Although it is too difficult to control the liquid nitrogen conditions, it is an efficient way of forming spherical shapes. The spherical shape is ideal, relative to other shapes, for filling in empty spaces without closed pores. It was noted in this experiment that spherical granules which have various pore volumes can be produced by varying the HAp-to-water ratio. Furthermore, the internal pore and micro-channel structures of various spherical HAp granules could be achieved by the addition of NaCl. When small amounts of NaCl were added to HAp slurry, the pore volume increased without changing the internal pore structure. However, when more than 15 wt% of NaCl was added to the HAp slurry, the pore volume decreased and caused a change in the internal pore structure into an irregular shape, as the NaCl resulted in grain formation. By changing the components of the HAp slurry, the pore volume and micro-channel structure of the HAp granules also changed, and these have an effect on sustaining the drug release rate.

Porous spherical HAp granules should be applied to bone grafts as bone filler. These HAp granules can function as a drug carrier due to their nano-, micro-interconnected and micro-channel pores.

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