Calcium phosphate cements comprising spherical porous calcium phosphate granules: synthesis, structure, and properties

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ABSTRACT
Calcium phosphate cements (CPCs) are used as artificial bone materials. The bone regeneration ability of CPCs can be improved by controlling their composition, porosity, and pore size. This study aims to design novel CPCs with high bone regeneration ability by controlling their microstructure. CPCs with macropores and micropores were prepared by incorporating spherical porous calcium phosphate granules composed of rod-shaped, calcium-deficient hydroxyapatite (CDHA) or plate-shaped octacalcium phosphate (OCP) particles. The granules were mixed with a binder (cement powder) composed primarily of α-tricalcium phosphate. The structure, morphology, compressive strength, porosity, specific area, pore size distribution, dissolution characteristics, and effects on cell viabilities were studied for the synthesized samples. The CPCs composed of porous granules had high porosity (~80%) and both macropores and micropores, which are expected to contribute to bone regeneration. The CPCs composed of porous granules showed a smaller specific surface area but a larger dissolution rate than the granule-free samples. The CPC composed of OCP granules showed a higher dissolution rate than the CPCs containing CDHA granules. In the cell culture experiments, the preosteoblasts proliferated on the CPCs, indicating that these CPCs could function as scaffolds for bone regeneration.

1. Introduction
Bone is an important organ that supports our bodies. Graft implantation is required when an injury or disease causes large bone defects. Autologous bone grafts are preferentially used because of their excellent bone regeneration abilities, but their availability is limited [1,2]. Calcium phosphate ceramics are widely used as artificial materials for bone repair [3–5]. Hydroxyapatite (Ca_{10}(PO_4)_6(OH)_2, HA) is the main inorganic component of bones and teeth, and HA ceramics are used as artificial bone substitutes because HA can bond to the viable bone tissue [6,7]. Other calcium phosphates, such as β-tricalcium phosphate (Ca_3(PO_4)_2, β-TCP) [8–10] and octacalcium phosphate (Ca_8(HPO_4)_2(PO_4)_4·5H_2O, OCP) [11,12], are also used as bone graft materials.

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Among calcium phosphate materials, calcium phosphate cements (CPCs) have received considerable attention because of their injectable properties [13–15]. CPCs are essentially pastes of a mixture of calcium phosphate powders and aqueous solutions. The paste is filled into the defects and placed directly in situ. The advantages of CPCs include easy handling and filling of complex-shaped defects. To enhance the bone regeneration ability of CPCs, their composition, porosity, and pore size should be properly controlled. For designing biore- sorbable bone substitutes, high porosity and good pore size control are required. Macropores with a diameter larger than 100 μm for the entrance of cells and vessels and micropores with a diameter less than 1 μm for the entrance of body fluid and the increase in surface area are needed [16]. Based on the CPCs setting mechanism, micropores are formed between the precipitated particles; however, macropores are not present in the conventional CPCs. Several methods have recently been developed to produce high porosity and macropores in CPCs by mixing soluble porogen agents into CPC [17–19] or using gas-generated foams [20–22]. These methods can easily make CPCs highly porous, but insufficient pore connectivity and adverse effects of the additives remain an area of concern.

Recently, CPCs with macropores and micropores have been prepared by combining porous granules [23,24]. Porosity can be increased by controlling spaces between the granules and by using granules with high porosity. Macropores are formed because the spaces between the granules and micropores can be modified by controlling the microstructure of the granules. Porous spherical granules of Ca-deficient HA (CDHA) [25,26] and OCP/HA [27–29] have been prepared in earlier studies. Previous studies have found that CDHA composed of rod-shaped particles exhibits high osteoconductivity with mild biodegradability [30] because of specific protein adsorption [31]. The CDHA also has the potential to serve as a drug delivery carrier [32]. OCP and OCP/HA were also found to support bone regeneration [11,12,33].

Here, we propose the design of novel CPCs that show high bone regeneration ability by controlling their microstructure using porous spherical granules which are expected to high bone regeneration ability. CPCs were prepared by incorporating porous spherical granules of CDHA and OCP and binders (Figure 1) to create the macropores and micropores. The porous spherical granules were connected to each other with binders whose compositions were similar to those of the commercial CPCs. The microstructures, mechanical properties, dissolution characteristics, and cell proliferation properties of the prepared samples are presented in this study.

2. Materials and methods
2.1. Preparation of porous spherical granules of CDHA and OCP

Porous spherical granules of CDHA and OCP were prepared using the hydration process of porous spherical granules composed of α-tricalcium phosphate (Ca₃(PO₄)₂, α-TCP).

The porous, spherical α-TCP granules were prepared as follows: 28 g of α-TCP powder (Taihei Chemical Industrial Co., Ltd., Japan) was dispersed in 140 g of 1.5 mass% agarose solution (Agarose S, Nippon Gene Co., Ltd, Japan). The resultant slurry was dispersed by stirring in 2 dm³ of vegetable oil, which was preheated at 60°C for 10 min. The oil was then cooled with ice water to set the agarose. Spherical granules were collected, washed with ethanol, and dried under ambient conditions. The dried granules were heated at 1200°C for 30 min to produce the spherical-TCP granules. The spherical porous α-TCP granules with diameters ranging from 300 μm to 500 μm were collected using sieves.

CDHA granules were prepared using the hydrothermal synthesis method based on our previous paper [34]. The obtained α-TCP granules (1.0 g) were placed in an 80-cm³ Teflon®-lined autoclave along with 20 cm³ of distilled water. In this setting, the granules were placed on the Teflon® stage and were not in direct contact with the water. The autoclave was placed in an oven heated to 105°C, and the granules were hydrothermally treated with water vapor at 105°C for 24 h. The treated granules were washed with water to obtain CDHA granules.

OCP granules were also prepared by the hydrolysis of α-TCP granules based on the previous paper [28]. Acetic acid-sodium acetate buffer solution (pH 5.0) was prepared by mixing the solutions of 0.1 M acetic acid and 0.1 M sodium acetate at 60°C. The 0.1 M acetic acid and 0.1 M sodium acetate were prepared by dissolving the acetic acid (CH₃COOH, 99.7%, FUJIFILM Wako Pure Chemical Corporation, Japan) and sodium acetate trihydrate (CH₃COONa·3H₂O, 99.0%, FUJIFILM Wako Pure
2.2. Preparation of CPCs with controlled microstructure

The binders for CDHA granules were obtained by mixing powdered α-TCP, tetracalcium phosphate (Ca₄(PO₄)₂O, TTCP, Taihei Chemical Industrial Co., Ltd.), and calcium hydrogen phosphate dihydrate (CaHPO₄·2H₂O, DCPD, 98.0%, FUJIFILM Wako Pure Chemical Corporation) in the ratio α-TCP:TTCP:DCPD = 75:20:5 (mass%). This ratio was derived from the conventional CPC composition [35]. By contrast, the binders for OCP granules were obtained by mixing powdered α-TCP and TTCP in the ratio α-TCP:TTCP = 80:20 (mass%). The Ca/P molar ratio of OCP (1.33) was lower than that of α-TCP (1.50), and the Ca/P molar ratio of the binder for OCP granules was designed to be higher than that of CDHA granules.

An aqueous solution of sodium alginate was used to enhance the aggregation properties of the paste [36]. Sodium alginate (80 ~ 120 cp (10 g/dm³), FUJIFILM Wako Pure Chemical Corporation) was dissolved in distilled water to obtain a 1.0 mass% sodium alginate aqueous solution. The granules and binder powders were mixed in specific ratios, as listed in Table 1. The mixtures and 1.0 mass% sodium alginate aqueous solution were mixed in a powder/liquid ratio (P/L) of 1.2/1 and placed in a Teflon® mold (diameter 6 mm, height 12 mm). The names and compositions of CPC are listed in Table 1. As a reference, the HA0-100 sample was prepared without mixing the granules. The CPC pastes were kept in 100% humidity at 37°C for 1 h and then in distilled water at 37°C for up to 72 h.

2.3. Properties of CPCs

Setting times of the CPC samples were obtained using a Gillmore needle apparatus (40DP-63L0075, Ogawa Seiki Co., Japan). The kneaded cement and paste were filled in a mold (diameter 6 mm, height 4 mm); after mixing for 2 min, they were placed in an incubator at 37°C and 100% relative humidity, and a sample was taken and assessed every 1 min. The needle was gently placed on the sample surface using the Gillmore needle apparatus, and we measured the time elapsed for no indentation to form. The weight and diameter of the Gillmore needle were 113 g and 2.12 mm, respectively. The setting time tests were performed on the samples HA70-30, OCP70-30, and HA0-100.

Properties of the CPC samples, except for the setting time test, were examined for the samples that had been kept in 100% humidity at 37°C for 1 h and then in distilled water at 37°C for up to 72 h. The crystal phase of the samples was examined by XRD. Fractured surfaces of the set CPCs were observed using SEM. Compressive strength was measured by performing a compressive test under wet conditions using a mechanical testing machine (EZ-L, Shimadzu Corporation, Japan). The compressive strengths of three specimens were measured for each sample. Porosity was determined from the ideal density and bulk density, calculated from the weight and size of the three specimens. The specific surface area was measured using the N₂ gas adsorption method (Autosorb-iQ, Anton Paar, Austria). Pore size distributions were obtained using the mercury intrusion method (AutoPore V 9620, Micromeritics Instrument Co., USA).

Dissolution characteristics were examined by measuring the dissolution rate. According to a previous report, a 0.08-M acetate buffer solution (pH 5.5) at 25°C was used for the dissolution [37]. The buffer solution was prepared from acetic acid and sodium acetate. The samples (100 mg) were placed in a polyethylene net and soaked in 100 cm³ of the 0.08-M acetate buffer solution (pH 5.5) at 25°C. The solution was stirred during the dissolution test, and the dissolved Ca²⁺ concentrations were measured using a Ca²⁺ electrode (Horiba Ltd., Japan) for up to 30 min.

Table 1. Composition of samples.

| Sample | Phase of granule | Mass ratio of granule:binder | Solids (g)/Liquid (cm³) |
|--------|-----------------|------------------------------|-------------------------|
| HA60-40 | CDHA            | 60:40                        | 1.2                     |
| HA70-30 | CDHA            | 70:30                        | 1.2                     |
| HA80-20 | CDHA            | 80:20                        | 1.2                     |
| OCP70-30 | OCP             | 70:30                        | 1.2                     |
| HA0-100 | -               | 0:100                        | 1.2                     |
2.4. Cell viability and proliferation on CPCs

Cell viability and proliferation were measured in vitro. The preosteoblast line MC3T3-E1 was purchased from the RIKEN Bioresource Research Center. The MC3T3-E1 cells were cultured in α-Minimal Essential Medium containing 10 vol% fetal bovine serum and the recommended concentration of penicillin–streptomycin (all obtained from Thermo Fisher Scientific, USA). Specimens (diameter 6 mm, width 3 mm) of the samples HA70-30 and OCP70-30 were used for the cell culture tests. A specimen (5 mm × 5 mm × 3 mm) of the commercially available porous HA ceramic (APACERAM-AX, Hoya Technosurgical, Japan) was used as the reference material. Five specimens were used for each test. The specimens were sterilized using ethanol and heat treatment at 105°C. The cell suspensions (0.5 cm³) containing 6 × 10⁴ cells/cm³ were inserted into a 48-well plate containing the specimens. After a 3-day and 7-day incubation period, cell activity was estimated using the Cell Counting Kit-8 (Dojindo Laboratories, Japan). The 7-day incubated specimens were fixed with 10% formalin neutral buffer solution and dehydrated with ethanol. The specimens were dried, and their fractured surfaces were observed using SEM. In the SEM observations, a Pt coating was applied to the specimens.

2.5. Statistical analysis

The data were statistically evaluated by the analysis of variance (ANOVA) and the Tukey test.

3. Results

The setting times of the HA70-30, OCP70-30, and HA0-100 samples were 36 ± 1, 22 ± 1, and 40 ± 1 min, respectively. All the other samples were also set within 1 h in 100% humidity at 37°C.

Figure 2 shows the XRD patterns of the CDHA and OCP granules used for the cements. The XRD patterns show that the CDHA granules were mainly composed of the HA phase, with a small amount of the β-TCP phase. In our previous paper [34], we prepared spherical CDHA granules under similar conditions. We showed the Ca/P molar ratio of the spherical CDHA granules prepared from α-TCP granules at 105°C was 1.55. Therefore, the Ca/P molar ratio is speculated near 1.55, and CDHA is presumed to have formed. The OCP granules were primarily composed of the OCP phase, with a very small amount of the HA phase. The SEM images (Figure 3) show that both the CDHA and OCP granules were spherical and porous. The CDHA and OCP granules were composed of rod-shaped and plate-shaped particles, respectively.

The XRD patterns of the set CPC samples HA70-30 and OCP70-30 are shown in Figure 4. HA70-30 was mainly composed of HA and a small amount of β-TCP, whereas OCP70-30 was composed primarily of OCP and HA.
Table 2 lists the compressive strengths of the samples. The compressive strengths of the granule-containing samples HA60-40, HA70-30, HA80-20, and OCP70-30 were ~1 MPa or less. The compressive strength of HA0-100 as the reference material was much higher than the granule-containing samples, and statistical analysis was conducted among the granule-containing samples. Among the samples containing CDHA granules, the compressive strength decreased with decreasing binder amount. The compressive strength was remarkably low for the sample with a low binder content, namely, HA80-20.

Table 3 lists the porosities and specific surface areas of the samples. The porosity of HA0-100 was ~50%, whereas that of HA60-40, HA70-30, HA80-20, and OCP70-30 was ~80%. The porosity of HA0-100 as the reference material was much smaller than the granule-containing samples, and the statistical analysis was conducted among the granule-containing samples. The porosity increased owing to the incorporation of the spherical porous granules. By contrast, the specific surface area decreased with the incorporation of the granules. Results of the pore distribution measurement for HA70-30, OCP70-30, and HA0-100 are shown in Figure 6. Although HA0-100 did not have macropores with diameters ~100 μm, the HA70-30 and OCP70-30 samples had macropores and micropores with diameters ~100 μm and 1 μm, respectively. The results of the pore size distribution measurements were consistent with those of the SEM observations.

Figure 7 shows the changes in the Ca²⁺ concentrations of the solution during the dissolution rate measurement of samples. The Ca²⁺ concentrations increased with time for all the samples. The increase in the Ca²⁺ concentrations indicates the dissolution of the samples because the samples are composed of calcium phosphates. The dissolution rate can be derived by calculating the rate at which the Ca²⁺ concentrations increase. The granule-containing samples showed higher dissolution rates than the granule-free sample HA0-100. For the samples containing CDHA, the dissolution rate increased with increased spherical granule content. OCP70-30 exhibited a higher dissolution rate than the samples containing CDHA granules.

Figure 8 shows the cell activity of the MC3T3-E1 cells on the samples. Considering the microstructure and strength, HA70-30 and OCP70-30 were selected for test evaluation. The absorbance is related to the metabolic activity of the cells. Comparing the activity between 3 and 7 days, the activity increased with time, indicating that the cells proliferated on all the samples. HA70-30 and OCP70-30 acted as scaffolds for the cells like the commercially available HA porous ceramics. The SEM images of the fractured surfaces of incubated samples from day 7 show that the cells were present on the inner surfaces of the samples (Figure 9).
4. Discussion

CPC composed of porous spherical granules exhibited the expected structures, as shown in Figure 1. Macropores with diameters larger than $\sim 100$ μm were present because of the formation of spaces between the granules, and micropores with diameters $\sim 1$ μm and less were present due to the pores present in the granules and binders. The size and volume of the macropores can be controlled by varying the size of the granules and the amount of binder added, respectively. Although when a smaller amount of binder was added, the number of macropores increased, and the compressive strength decreased. To balance the microstructure and strength, HA70-30 was expected to be a suitable sample for this study. The micropores

Figure 6. Pore distribution measured by the mercury intrusion method for the samples HA70-30, OCP70-30 and HA0-100.

Figure 7. Changes in Ca$^{2+}$ concentrations during the dissolution rate measurement.

Figure 8. Cell activity of MC3T3-E1 cells on samples HA70-30 and OCP70-30, compared to HA ceramics. *There is no significant difference at the 5% significance level between values marked with the same symbol among the samples.
can be controlled by controlling the microstructure of the granules. In this study, both CDHA and OCP granules were used to design CPC. High porosity CPC can also be obtained using high porosity granules. Honda et al. [38] reported using bactericidal and bioresorbable calcium phosphate cement using silver-containing tricalcium phosphate microspheres whose diameters were 0.5–3 μm. However, these cement types using small microspheres are not expected to provide macropores. Compared to previous studies [23,24], the advantage of this cement is that various granules with high bone regeneration ability can be selected. Moreover, the cements used in the current study are constructed with calcium phosphates without the use of porogens or foaming agents, which may adversely affect the body; it is safer to avoid their use.

Although the CPCs with porous spherical granules had low compressive strength owing to their high porosity, the compressive strength was comparable to that of the highly porous HA ceramic used in clinical applications, and they can be used in the non-loaded part of bone regeneration, for example, small defects or parts supported by fixation devices. Le et al. [39] reported on porous CDHA microspheres laden with brushite-based injectable cement, but CDHA microspheres are fully embedded in the cement, and macropores among the microspheres are not expected. For the non-loaded part of bone regeneration, the macropores are expected to be more important than the mechanical strength.

The specific surface areas of the samples decreased with the incorporation of the granules because the size of the constituent particles of the granules is much larger than that of the particles precipitated from the binders. For the samples containing CDHA granules, the dissolution rate increased with an increase in spherical granules. Although the dissolution rate was expected to be related to the specific surface area, the specific surface areas of the CPC samples decreased with an increase in spherical granules. The dissolution rate increased due to the macropores and incorporated granules in the samples. Since bulk specimens were used in the dissolution rate test, it was difficult for the dissolved ions to diffuse from inside the specimens without macropores. However, the macropores formed by the spherical granules functioned as a pathway for the buffer solution, such that the buffer solution efficiently reached the inside of the sample, increasing the surface involved in the dissolution reaction. OCP70-30 exhibited a higher dissolution rate than the samples containing CDHA granules, indicating that the properties of the granules also affect the dissolution rate. The solubility of OCP is higher than that of HA [14], and the high solubility of OCP in granules could be contributing to the increased dissolution rate.

Due to difficulty in evaluating porous materials, such as the harvest method, the precise control of the cell numbers is difficult; however, because the cell numbers increased with the culture period, CPC did not demonstrate cell toxicity or inhibit cell growth. As the CPC prepared in this study were composed of CDHA and OCP, they are expected to be incorporated into the bone matrix to induce bone regeneration.

5. Conclusions

In this study, CPCs composed of porous spherical CDHA or OCP granules were prepared to obtain novel CPCs with a high bone regeneration ability. CPCs were prepared with designed macropores and micropores by binding the porous spherical CDHA or OCP granules with a binder consisting of mainly α-TCP. The volume of the macropores was controlled by the amount of the binder added. Synthesized CPCs possessed high porosities and solubilities. The CPC containing OCP granules exhibited a higher dissolution rate than the CPCs containing CDHA granules. No cell toxicity or cell growth inhibition effect of the prepared CPCs was observed regardless of the kind of granule used. The obtained CPCs are expected to be useful novel bone cements for bone regeneration.

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Disclosure statement

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