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Preparation and Characterization of Calcium Phosphate Cement of α-Tricalcium Phosphate-Tetracalcium Phosphate-Dicalcium Phosphate System Incorporated with Poly(γ-glutamic acid)

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Abstract: Polymeric calcium phosphate cements (PCPCs) were prepared from cement powders consisting of alpha-tricalcium phosphate (α-TCP), tetracalcium phosphate (TTCP) and dicalcium phosphate (DCP), and cement liquid with poly(γ-glutamic acid) (γ-PGA). The characteristics of calcium phosphate cements (CPCs), such as paste injectability, initial setting time, and compressive strength, were assessed. The injectability of PCPCs with a 0-30 wt% TTCP/DCP mixture was above 90%. The initial setting time decreased in concert with the amount of incorporated TTCP/DCP mixture and reached approximately 10-13 min when the amount of TTCP/DCP mixture was 10-30 wt%. The compressive strength of PCPC with the 30% equimolar TTCP/DCP mixture was approximately 18.4±3.5 MPa, whereas that of the CPC without the TTCP/DCP mixture was approximately 4.8±0.1 MPa.

Keywords: calcium phosphate cement, poly(γ-glutamic acid), tricalcium phosphate, tetracalcium phosphate.

Introduction

Calcium phosphate ceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP) have been used for bone replacement and augmentation due to their good biocompatibility and osteoconductivity. They are usually applied in the form of blocks or granules. However, difficulty exists in shaping blocks prior to surgery and in making them fit with the bone surface. These problems do not happen with granules, but secondary migration is often observed and mechanical strength comparable to blocks cannot be achieved. Recently, much attention has been paid to calcium phosphate cements (CPCs), because they can be handled in the paste form and set *in situ,* and they have the potential to overcome the practical disadvantages experienced with blocks or granules.[1-3]

CPCs have been utilized since 1983.[4] With the development of minimally invasive surgical methods, CPCs have received increasing interest in biomedical applications for repairing hard tissues in orthopedics, dentistry, and drug delivery due to the following advantages: 1) the ability to be injectable, 2) the ability to be used under ambient conditions, 3) the ability for self-setting through a low exothermic reaction in an aqueous environment, and 4) excellent biocompatibility due to their compositional similarity to bone and teeth.[5,6] Currently, CPCs are mainly applied for the treatment of maxillofacial defects and deformities as well as fracture defects.[7-9]

CPCs are obtained by mixing one or several reactive calcium phosphate powders with an aqueous solution to form a paste that hardens through a dissolution-precipitation mechanism within a restricted period of time.[10-13] During implantation, the cement paste is meant to harden through crystal entanglement even if it comes in contact with streaming blood or body fluids. Before being delivered to the defected site, however, the paste should be in a slurry state for convenient injection and should be able to maintain its integrity when it comes in contact with blood or body fluids. Disintegration of CPC due to contact with body fluids is an obstacle for its wide application.

We have previously studied a polymeric calcium phosphate cement (PCPC) derived from alpha-tricalcium phosphate (α-TCP) and poly(γ-glutamic acid) (γ-PGA), which are nontoxic, biodegradable, and water-soluble and have numerous negatively-charged carboxylic groups.[14,15] γ-PGA improved cement integrity, paste viscosity, cement injectability and set-

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ting property. However, compressive strength was shown to decrease with the incorporation of γ-PGA.

The α-TCP/tetracalcium phosphate (TTCP)/dicalcium phosphate (DCP) system was originally developed by Monma *et al.* and reported to be proper for orthopedic application.[16] In the present study, we studied the effects such as handling and mechanical properties of the α-TCP/TTCP/DCP system incorporated with γ-PGA. According to their extensive studies, this cement-type material can be refined, demonstrating better biocompatibility and direct integration to bones without any participation of peripheral soft tissues.[16-19] Additionally, the cement system incorporated with sodium chondroitine sulphate showed high compressive strength.[17]

Experimental

Preparation of Calcium Phosphate Cements. Dicalcium phosphate ($CaHPO_4$, Sigma Chemical) and calcium carbonate ($CaCO_3$, Sigma Chemical) were used at a 2:1 molar ratio as starting materials for the fabrication of α-TCP [$Ca_3(PO_4)_2$]. The batch mixture with a Ca/P molar ratio of 1.5 was homogenized by ball milling in ethanol for 24 h and then dried. The dried powder was calcined at 1,300 °C for 12 h in an electronic furnace and then quenched at room temperature. TTCP was also synthesized from a solid-state reaction between $CaHPO_4$ and $CaCO_3$ with a Ca/P molar ratio of 2.0, which were mixed and heated at 1,400 °C for 12 h in the furnace. The heated mixture was quenched to room temperature. The lumps of α-TCP and TTCP were first crushed by a mortar and pestle respectively and then wet ground in a planetary ball mill for 12 h. Absolute ethyl alcohol was used as the medium liquid. After drying the ground materials, they were sieved using a sieve with an aperture size of 63 μm. Dicalcium phosphate anhydrous (DCP) was prepared by baking commercial DCPD (Sigma Chemical) at 120 °C, followed by the same wet grinding processing.

To make cement powders, the mixture of TTCP and DCP in an equivalent molar ratio was first made. The cement powders were prepared by mixing the α-TCP powder and the mixture of TTCP and DCP, whose weight ratios were 0, 10, 20, 30, 50, and 100 wt%. Cement liquid was in the form of aqueous solution with 2.5 wt% sodium hydrogen phosphate (Sigma Chemical) and 5 wt% γ-PGA (MW=about 500 kg/mol; BLS 50, Bioleaders Co., Korea).

To make the cements, the cement powders and liquids were added together at a 10:4.5 weight-volume ratio (10 g/4.5 mL) in a polytetrafluoroethylene (PTFE) beaker and mixed manually with a spatula for approximately 1 min. The prepared cement paste was then cured in physiological saline solution in an incubator at 37 °C to simulate a physiological environment.

Characterization of Calcium Phosphate Cements. The morphology of the prepared α-TCP and TTCP particles was examined by scanning electron microscopy (SEM, JSM-840A, JEOL Ltd.) and transmission electron microscopy (TEM, JEM-2010, JEOL Ltd.). The particle size and the distribution of the particles were measured using a particle size analyzer (BI-DCP, Brookhaven Instruments Ltd.). The formation of α-TCP and TTCP was assessed by powder X-ray diffraction (XRD) analysis. The XRD pattern was recorded on a Rigaku Rotaflex diffractometer (D/Max-2200 Ultima/PC, rotating Cu target, 3 kW X-ray and set to 40 kV and 40 mA) at an X-ray incident angle of 0.02°. The sample was scanned 20-40 degrees in 2θ using the step-scanning mode with an integration time of 10 s at intervals of 0.02°. Peak indexing was carried out by means of cards: JCPDS-29-359 for α-TCP, JCPDS-9-169 for β-TCP and JCPDS-9-432 for apatite (Joint Committee on Powder Diffraction Standards, 1988). The percentage of α-TCP in the particles was estimated according to our previous study.[14]

The injectability of CPC pastes was tested with a 10 mL syringe fitted with a 14G needle. The cement paste was transferred into the syringe and the syringe was put into a tube-type jig.[20] The paste was then extruded using a mechanical testing machine (Instron 4482, Illinois Tool Works Inc) at a cross-head speed of 20 mm/min and a maximum force of 300 N. This force was selected since this was the maximum force that could be applied manually. The amount of cement remaining within the syringe was weighed and injectability (I) was calculated as follows:[21]

$$I\,(\%) = \frac{\text{Mass of cement paste injected through needle}}{\text{Original mass of cement paste in syringe}} \times 100$$

The effective surface charges of CPC powders in contact with the cement liquids were determined from zeta-potential measurements. Electrophoretic light scattering measurements (ELS 8000, Otuska electronics) were analyzed. Measurements were performed 5 times and the average potential and standard deviation were calculated.

The initial setting times of the cement pastes were measured using the Gilmore needle method. Three wells of a 24-well tissue culture plate were filled with each cement paste. The plate was kept in physiological saline solution at 37 °C to simulate a physiological environment. The sample was considered set when a 100-g mass loaded on a needle with a tip diameter of 1 mm failed to make a perceptible circular indentation on the surface of the cement.

The compression test of wet CPC samples cured in physiological saline solution for 1 week was carried out according to ASTM F451-86 recommendations. The compression load was applied along the axis using a crosshead speed of 1 mm/min. In the mechanical tests, the data were expressed as mean and standard deviation.

Following the mechanical tests, the fractured surfaces were observed by SEM after the samples had been coated with platinum. Fourier transform infrared (FTIR) spectroscopy (EQUINOX 55, Buker) and XRD were performed to determine the chemical reactions and material formation.

Results and Discussion

The prepared α-TCP particles were irregularly shaped when observed by SEM and TEM (data not shown). α-TCP was 15.5±0.5 μm by particle size analysis and it was impossible to obtain pure α-TCP. The second phase was the polymorphic form of tricalcium phosphate, β-TCP, and was in the stable phase at low temperatures, T<1,180 °C. At higher temperatures, α-TCP was in the stable phase. It is well known that during the quenching process, some α-TCP transforms into β-TCP, which is difficult to avoid. The prepared α-TCP particles were estimated to include about 23.2±6.3% of the β-TCP phase based on XRD analysis.[14,15]

Figure 1 shows the typical load/displacement diagrams of injectability experiments. CPC without the equimolar mixture of TTCP and DCP (TTCP/DCP mixture) was injectable with a small load for about 30 mm displacement after the beginning of injection; the load then increased and reached 300 N. The injection behavior of CPC with the 20 wt% TTCP/DCP mixture was similar to that of CPC without the mixture. However, when the incorporation of TTCP/DCP mixture was above 50 wt%, the applied load increased at the beginning of injection as shown in Figure 1 (TTCP/DCP=50%).

Figure 2 shows the changes of injectability with increasing incorporation of the TTCP/DCP mixture in the cement powders. The injectability of CPCs with the 0-30 wt% TTCP/DCP mixture was above 90%, which was not entirely dependent on the incorporation of the TTCP/DCP mixture. However, lower injectability of CPCs was detected with above 50 wt% of the TTCP/DCP mixture. The injectability of CPCs with 50 and 70 wt% TTCP/DCP mixtures was 78% and 70%, respectively.

Figure 3 shows the changes of zeta potentials of CPC powders in contact with the cement liquid (aqueous solution with 2.5 wt% sodium hydrogen phosphate and 5 wt% γ-PGA)

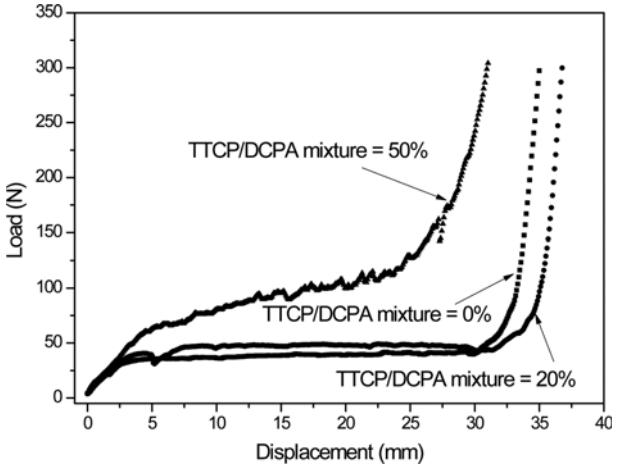

Figure 1. Typical load/displacement diagrams of injectability experiments with CPCs prepared with 0, 20, and 50 wt% equimolar TTCP/DCP mixtures; crosshead speed 20 mm/min, Powder weight/Liquid volume=10/4.5 g/mL.

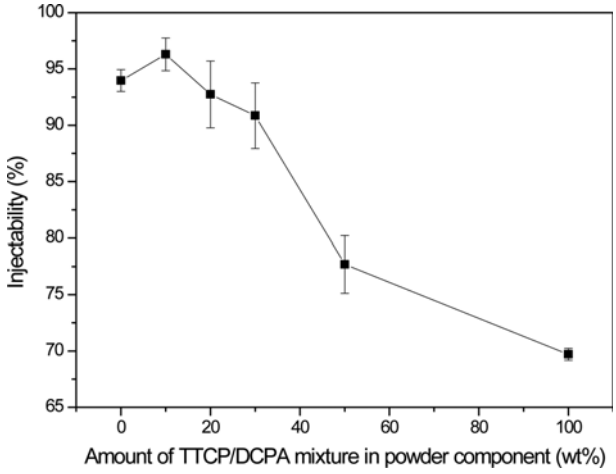

Figure 2. The cement injectability versus the amount of equimolar TTCP/DCP mixtures in the powder components.

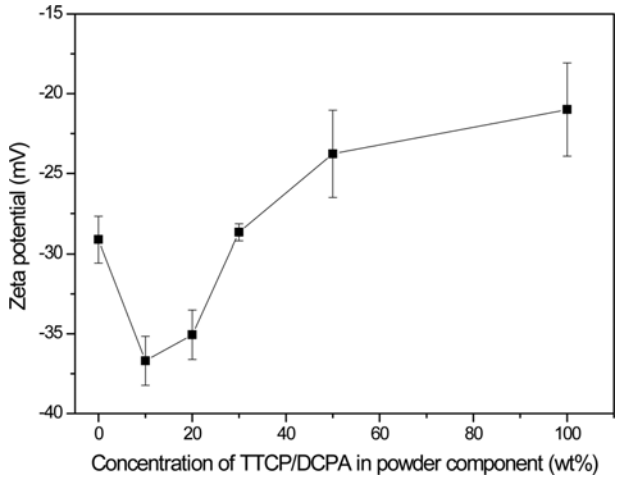

Figure 3. The zeta potential of cement powders versus the amount of equimolar TTCP/DCP mixtures in the powder components.

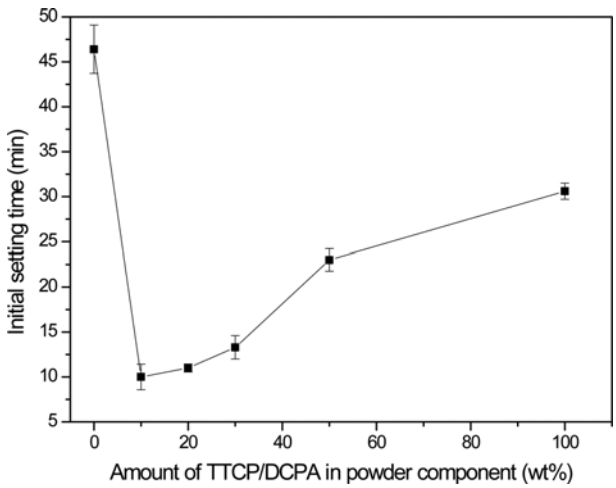

Figure 4. The initial setting time versus the amount of equimolar TTCP/DCP mixtures in the powder components.

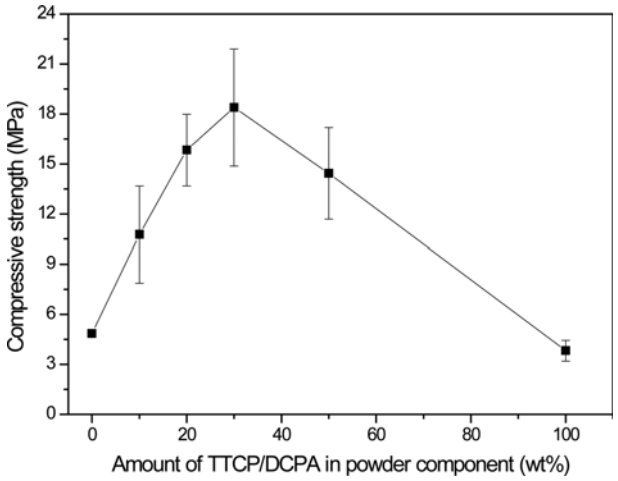

Figure 5. The changes of compressive strength of the set cements (incubation time=1 week) by the amount of equimolar TTCP/DCP mixtures in the powder components.

with increasing incorporation of the TTCP/DCP mixture. The zeta potentials of CPC powders with the 0~30 wt% TTCP/DCP mixture were about -29 ~ -36 mV. The zeta potential became more positive with increasing incorporation of the TTCP/DCP mixture and thus reached approximately -23 and -20 mV for CPC powders with 50 and 100 wt% TTCP/DCP mixtures, respectively.

Figure 4 shows the changes in the initial setting time with the increase in the amount of TTCP/DCP mixture in the cement powders. The initial setting time of CPC without the TTCP/DCP mixture was approximately 45 min. The initial setting time was decreased with increasing the incorporated TTCP/DCP mixture and reached approximately 10~13 min when the amount of TTCP/DCP mixture was 10~30 wt%. However, it was increased when the amount of TTCP/DCP mixture was above 50 wt%.

Figure 5 shows the compressive strength (CS) of cements incubated in physiological saline solution for 1 week. The CS of CPC without the TTCP/DCP mixture was approxi-

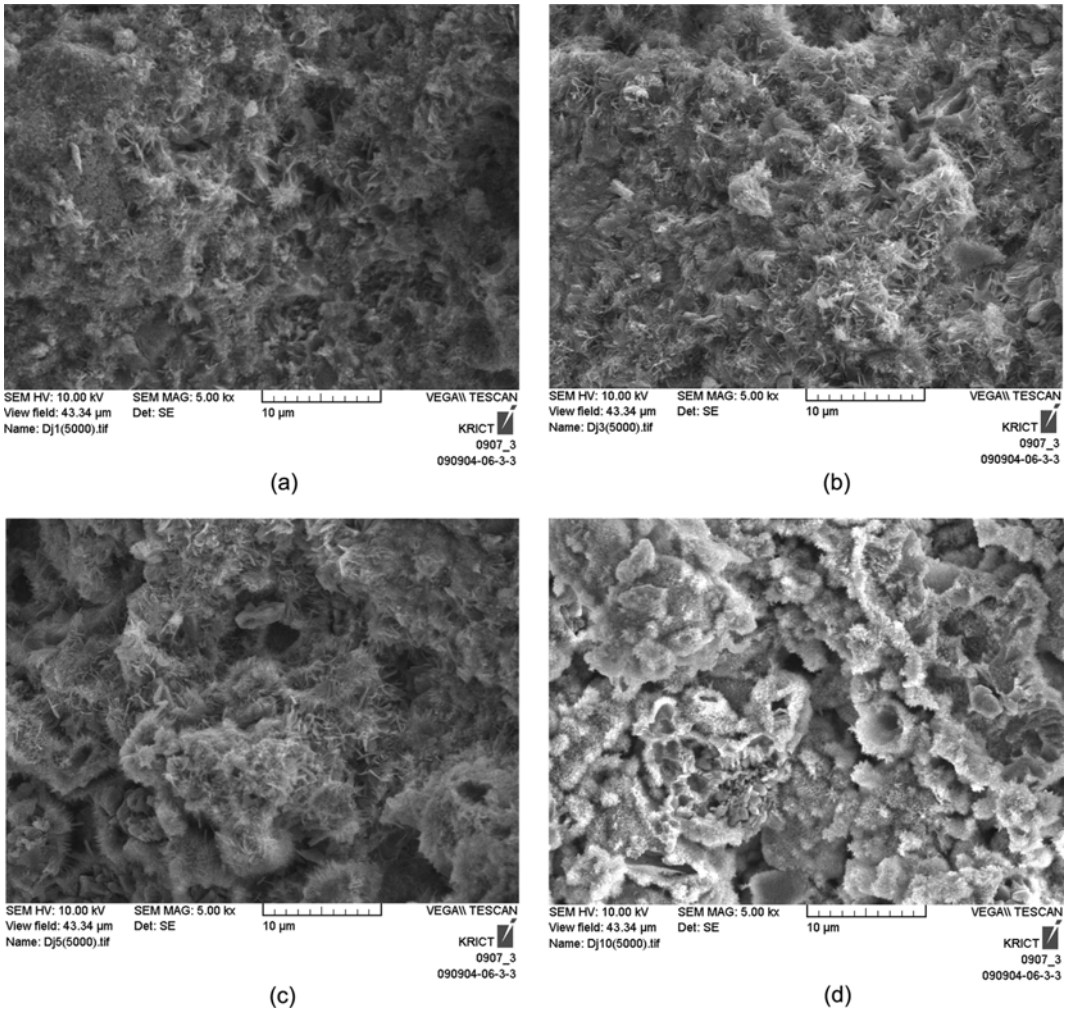

Figure 6. The SEM images (×5000) of calcium phosphate cements prepared with cement powders containing 0 (a), 30 (b), 50 (c), and 100 (d) wt% equimolar TTCP/DCP mixtures, after 1-week of incubation.

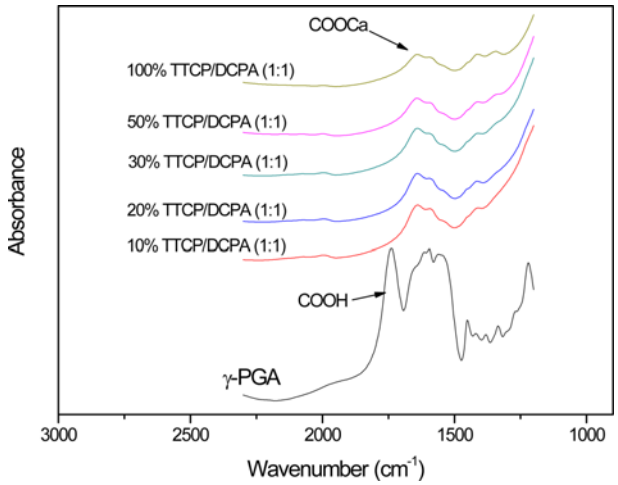

Figure 7. The FTIR spectra of the carboxyl stretching region of TCP cements incubated for 1 week. The spectra are those of set cements prepared with 10 (10% TTCP/DCP (1:1)), 20 (20% TTCP/DCP (1:1)), 30 (30% TTCP/DCP (1:1)), 50 (50% TTCP/DCP (1:1)), and 100 wt% (100% TTCP/DCP (1:1)) equimolar TTCP/DCP mixtures and γ-PGA as a reference material.

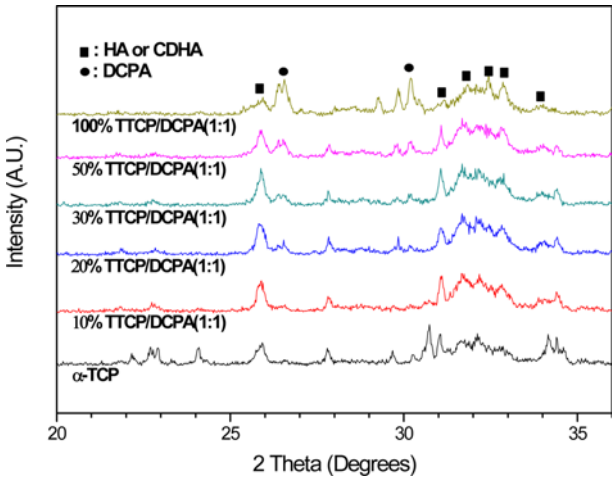

Figure 8. The XRD patterns of the prepared α-TCP powder and set cements prepared with powder components containing 10, 20, 30, 50, and 100 wt% equimolar TTCP/DCP, incubated for 1 week.

mately 4.8±0.1 MPa. It was increased with increasing the TTCP/DCP mixture and reached approximately 18.4±3.5 MPa when the amount of TTCP/DCP mixture was 30 wt%. Then, it was decreased with increasing the TTCP/DCP mixture when the amount of TTCP/DCP mixture was above 50 wt%.

Figure 6 shows the SEM micrographs of the fractured surface of the cements after 1-week of incubation. Needle-like formations, which are thought to be formed by HA generation,[22,23] were observed on all the surfaces.

Figure 7 shows the FTIR spectra of the carboxyl stretching region of γ-PGA and set cements that were incubated for 1 week. In the spectrum of γ-PGA, the carboxyl stretching peak of carboxylic acid appeared at 1736 cm^{-1}. However, in the spectra of set cements with γ-PGA, the carboxylic acid peak disappeared, but the carboxyl stretching peak of calcium acetate was observed at 1643 cm^{-1}.

Figure 8 shows the XRD patterns of the α-TCP powder and the set cements prepared with CPC powders containing different amounts of the TTCP/DCP mixture. The incubation time of the set cements was 1 week. Peaks of α- and β-TCP were detected in the α-TCP powder used in this study. Thus, the powder was considered to be a mixture of α- and β-TCP. In the XRD pattern of cement prepared using the CPC powders with different amounts of the TTCP/DCP mixture, the intensity of α-TCP peaks decreased and HA peaks appeared.

Hydroxyapaptite (HA) forms the main mineral constituent of human hard tissues. HA ceramics have proven to be biocompatible and bioactive and have been successfully used clinically in the repair of bone defects. CPC based on α-TCP forms HA in the human body as the following reaction:[24,25]

$$3\alpha\text{-}Ca_3(PO_4)_2+H_2O \rightarrow Ca_9(OH)(HPO_4)(PO_4)_5 \quad (1)$$

Another HA-forming CPC is the TTCP/DCP system. This system hardens through a setting reaction that converts an equimolar TTCP and DCP mixture to HA in an aqueous environment:[26]

$$Ca_4(PO_4)_2O+CaHPO_4 \rightarrow Ca_5(PO_4)_3OH \quad (2)$$

Monma *et al.*[16] and Fernandez *et al.*[27] have investigated regarding the α-TCP/DCP system. Kurashina *et al.* have studied about CPC consisting of α-TCP, TTCP, and DCP.[2] In these systems, octacalcium phosphate (OCP) and HA are produced by the following reaction:

$$2CaHPO_4+2\alpha\text{-}Ca_3(PO_4)_2+5H_2O \rightarrow Ca_8(HPO_4)_2(PO_4)_4\cdot 5H_2O \quad (3)$$

In our previous study, the α-TCP cement incorporated with γ-PGA showed improved cohesion strength and injectability of CPC.[14,15] However, its mechanical strength was not enough for clinical applications. In this study, the α-TCP/TTCP/DCP system was investigated by the incorporation of the TTCP/DCP equimolar mixture into the α-TCP cement consisting of γ-PGA to increase the mechanical properties, because the α-TCP/TTCP/DCP system is reported to show good compressive strength.[2] The effect of the equimolar mixture of TTCP and DCP on the main CPC properties such as injectability, initial setting time, pH, and compressive strength, *etc*. was studied in this study.

From the clinical point of view, a surgeon could take advantages of the improved cement injectability, provided that the injection is performed at an appropriate time after mixing of the cement. CPC injection is normally interrupted

by a filter-pressing phenomenon that leads to a phase separation of liquid and solid phases.[20] A high surface charge (or zeta potential) on the ceramic particles improves dispersion as a result of the mutual repulsion of the particles in the liquid phase.[20] In our previous study of α-TCP cement, the zeta potential of α-TCP particles became more negative with the incorporation of γ-PGA due to its carboxyl ions.[15] This high zeta potential of α-TCP particles in the solution with γ-PGA led to improved injectability of CPCs.

In this study, the zeta potentials of cement powders were measured with the addition of TTCP/DCP mixtures (Figure 3). The zeta potentials of cement powders became more positive with higher concentrations of TTCP/DCP mixtures. This observed pattern is presumably related to the addition of DCP particles. The solubility of DCP is the highest among those of cement powders because solubility product constants [-log (Ks)] of α-TCP, TTCP, and DCP are 25.5, 38-44, and 6.90, respectively.[24] Therefore, the DCP particles may show the highest release of Ca^{++} ions. Because Ca^{++} ions are positive, the increase in the amount of Ca^{++} ions leads to positive increase of zeta potentials. Therefore, more DCP incorporation in the cement system causes more release of Ca^{++} ions and thus the cement injectability may be decreased. However, injectability was higher than 90% when the amount of TTCP/DCP mixtures was 30 wt% or less.

In cement pastes, cement particles such as α-TCP and TTCP particles dissolve into calcium and phosphate ions through an acid-base reaction and then transform into a low-crystalline HA by the precipitation of the supersaturated ionic materials.[21] The cement pastes are then hardened through crystal entanglement of HA. Therefore, the setting of CPC is thought to result from crystal entanglement. The initial setting time is related to the time the cement settles down on a target site during clinical application. When it is too slow, the cement is washed out by the body fluid. On the other hand, when the initial setting time is too fast, the cement delivery becomes difficult. Generally, the optimal initial setting time is reported to be around 10 min.[10,13] The initial setting time of CPC with 10-20 wt% of the TTCP/DCP mixture was within the optimal range.

The main disadvantage of the known CPCs is their low mechanical strength, which is similar to that of trabecular bone, or one fifth of that of cortical bone. Therefore, the availability of CPCs with mechanical properties resembling those of human bones would considerably extend the potential fileds of application. In this study, CS was increased by the addition of TTCP/DCP mixtures. This strength increase is presumably due to HA-forming reactions. That is, HA crystals are formed by reactions (1)-(3) in the α-TCP/TTCP/DCP cement system prepared by the addition of the TTCP/DCP equimolar mixture, whereas in the α-TCP cement, HA is only generated from reaction (1). These different HA-forming reactions may increase the crystal entanglement. However, strength was much lower than those of human bone, because the tensile and compressive strengths of human compact bone are 121-149 and 114-167 MPa, respectively.[28] The mechanical strength of CPCs in this work may not be enough for applications to areas under great stress such as discontinuity defects of long bones and jaw bones.

SEM, FTIR, and XRD analyses were carried out to determine the chemical and physical behaviors in the set cements (Figures 6-8). According to SEM observations (Figure 6), large needle dimensions were similarly observed in all the cements. Tenhuisen and Brown[22] proposed that the needle dimension might be formed by HA generation. According to the FTIR spectra, in cements incorporated with γ-PGA, the carboxyl stretching region of carboxylic-acid groups disappeared during the cement-forming reaction, while the carboxyl stretching region of calcium carboxylate groups was observed (Figure 7). Therefore, most carboxylic-acid groups might react with Ca^{2+} ions to form ionic complexes such as calcium citrate and the γ-PGA-calcium complex. Similarly, the carboxyl stretching region of carboxylic-acid groups disappeared during the cement-forming reaction between TTCP and organic acids such as citric acid and acetic acid.[22] γ-PGA-calcium complexes may also form ionic crosslinks with a high degree of cross-linking because of the numerous carboxylic-acid groups in the γ-PGA molecule. The crosslinked polymer chains surround the unreacted TCP particles and thus increase the mechanical strength of CPC. According to the XRD patterns of the set cements, the specific peaks of HA appeared in all the set cements and the intensity of α-TCP and TTCP peaks decreased. Therefore, HA formation in the set cements was confirmed.

Conclusions

PCPCs were prepared from cement powders consisting of α-TCP, TTCP, and DCP, and cement liquid with γ-PGA, in an attempt to improve handling and mechanical properties. The injectability of CPCs with the 0-30 wt% TTCP/DCP mixture was above 90%. The initial setting time was decreased with increasing the incorporated TTCP/DCP mixture and reached approximately 10~13 min. CS was increased by the addition of TTCP/DCP mixtures.

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