MECHANOCHEMICAL SYNTHESIS OF ZINC CHLOROAPATITE AND EVALUATION OF ITS CRYSTALLINITY BY ATTENUATED TOTAL REFLECTION - INFRARED SPECTROSCOPY AND PRINCIPAL COMPONENT ANALYSIS

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Abstract: Zinc chloroapatite (ZnClAp), a promising material for bone treatment, was synthesized by a mechanochemical method. The effect of synthesis condition on its crystallinity was investigated. Starting materials (i.e., calcium monohydrogen phosphate dihydrate, calcium oxide, zinc oxide and calcium chloride) were kneaded with water at four kinds of water/solid ratios in a planetary ball-mill. Thus obtained products were analyzed by a powder X-ray diffractometry (PXRD) and an attenuated total reflection - infrared (ATR-IR) spectroscopy. The PXRD and ATR-IR results indicate that ZnClAp has successfully been synthesized by the present method. PXRD results indicate that the crystallinity of ZnClAp increases with the water/solid ratio of the kneaded paste for the synthesis. This finding is supported by a principal component analysis (PCA) that is applied to the ATR-IR spectra. The present mechanochemical method is simple, economy, and is useful for the synthesis of ZnClAp.

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INTRODUCTION

Synthesized hydroxyapatite (HAp) is one of the most commonly used biomaterials for bone treatment, because it has high mechanical strength and biocompatibility¹. Several papers on HAp-based treatment have been reported²-⁶. Higher crystalline HAp is, however, too stable for rapid bone replacement. Chloroapatite, Ca₁₀(PO₄)₆Cl₂ (ClAp) (pKᵢₛ = 108) is more soluble than HAp (pKᵢₛ = 116.8) and is expected to improve the bone replacement rate³⁷⁹. In the previous study¹⁰, ClAp was synthesized from CaHPO₄·2H₂O (DCPD), CaO and CaCl₂ by a mechanochemical method. The method was found to be useful for ClAp synthesis.

It is known that zinc ion regulates bone formation and bone resorption by promoting osteoblast and repressing osteoclast¹¹-¹⁸. Zinc-substituted hydroxyapatite, (Ca)₁₀₋ₓ(Zn)ₓ(PO₄)₆(OH)₂ (ZnHAp) retain its typical HAp characteristics despite the incorporation of Zn. In vitro cell culture work showed that ZnHAp possessed enhanced bioactivity since an increase in the growth of human adipose derived mesenchymal stem cells along with the bone cell differentiation markers was observed¹⁹. Considering the above findings, Zinc chloroapatite, (Ca)₁₀₋ₓ(Zn)ₓ(PO₄)₆Cl₂ (ZnClAp) is expected to have better solubility and bone metabolism ability than HAp.

Mechanochemical synthesis is a method for modifying crystal structure or producing new crystal based on the reaction between solid phases. Mechanical energy supplied through friction and compression initiates and promotes the reaction. The method has the following advantages: 1) simple operation, 2) high yield, 3) simple equipment, 4) less contamination, and 5) green nature (no organic solvent waste)²⁰. The method has been applied to the syntheses of various materials, such as an electrode material NaFeF₂, fullerene dimer C₁₂₀, caffeine/oxalic acid cocystal and nano photocatalyst TiO₂/Fe₂O₃. HAp can also been
synthesized mechanochemically from Ca$_2$P$_2$O$_7$, CaCO$_3$ and water.$^{25}$

The purpose of the present study is to mechanochemically synthesize ZnClAp and to evaluate its crystallinity by coupling spectroscopies with chemometrics. Starting materials (DCPD, CaO, ZnO and CaCl$_2$) are kneaded with water, then grounded by using a planetary ball-mill. The products are analyzed by a powder X-ray diffractometry (PXRD) and an attenuated total reflection - infrared (ATR-IR) spectroscopy. Principal component analysis (PCA) is applied to the ATR-IR spectra. The effect of storage in humid condition on the crystallinities of ZnClAp is investigated. The present mechanochemical method has been found useful for ZnClAp synthesis. The crystallinity of ZnClAp depends on the water/solid ratio before the synthesis and on the humidity of the ambient air after the synthesis.

EXPERIMENTAL

Materials
DCPD, CaO and ZnO were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). CaCl$_2$ was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). HAp was purchased from Nitta Gelatin Inc. (Osaka, Japan). Arium 611 DI deionized water was used throughout. Other reagents used in the present study were of analytical reagent grade and were used without further purification.

Methods
Mechanochemical synthesis
Synthetic method 1 (ZnClAp) : DCPD, CaO, ZnO and CaCl$_2$ were put in an agate jar of a planetary ball-mill (Pulverisette 7, Fritsch GmbH, Idar-Oberstein, Germany). The molar ratios of Ca to Zn and (Ca + Zn) to P were 19 : 1 and 1.67 : 1, respectively. The starting materials were kneaded with water at water/solid ratios of 0.5, 0.75, 1.0 and 1.2 mL/g. The pastes were respectively ground by the mill at a revolution speed of 1000 rpm. The set of the grounding (30 min) and interval (30 min) was repeated 12 times for 12 h. Thus obtained products (“Product A”) were poured into rubber molds (6 mm in diameter and 4 mm in height) and were dried in a drying oven (MOV-112, SANYO Electric Co. Ltd., Osaka, Japan) at 37°C for 24 h. It is considered that the reaction formula of Synthetic method 1 is as follows (Eq. 1).

$$6(\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}) + x\text{ZnO} + (3-x)\text{CaO} + \text{CaCl}_2 \rightarrow$$

$$(\text{Ca})_{10-a}(\text{Zn})_a(\text{PO}_4)_{3b}(\text{Cl})_2(\text{OH})_{2a} + (13+y)\text{H}_2\text{O} + (2-y)\text{H}^+ \quad (1)$$

Synthetic method 2 (ZnHAp) : The starting materials were DCPD, CaO and ZnO. The molar ratios of Ca to Zn and (Ca + Zn) to P were the same as those in Synthetic method 1. The starting materials were kneaded with water at the water/solid ratio of 1.0 mL/g. The set of the grounding (1000 rpm, 30 min) and interval (30 min) were repeated 24 times for 24 h. The product (“Product B”) was poured into the mold and dried at the same conditions as those for product A. The reaction formula The reaction formula of Synthetic method 2 is considered as follows (Eq.2).

$$6(\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}) + x\text{ZnO} + (4-x)\text{CaO} \rightarrow$$

$$(\text{Ca})_{10-a}(\text{Zn})_a(\text{PO}_4)_{3b}(\text{OH})_2 + 14\text{H}_2\text{O} \quad (2)$$

Synthetic method 3 (ClAp)$^{10}$ : The mixture of DCPD, CaO, CaCl$_2$ and pure water (Ca/P ratio, 1.67; water/solids ratio, 0.5 mL/g) was allowed to react in the ball-mill. The conditions of synthesis and drying were the same as those in Synthetic method 1. The reaction formula of Synthetic method 3 is expressed as follows (Eq.3).

$$6(\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}) + 3\text{CaO} + \text{CaCl}_2 \rightarrow$$

$$\text{Ca}_{10}(\text{PO}_4)_3(\text{Cl})_2(\text{OH})_{2a} + (13+y)\text{H}_2\text{O} + (2-y)\text{H}^+ \quad (3)$$

PXRD
The synthesized products were analyzed by PXRD with an X-ray diffraction instrument (MiniFlex, Rigaku Co. Ltd., Tokyo, Japan). Measurement conditions were as follows: scan type, continuous scan; divergence slit, variable; scattering slit, 4.2 deg.; receiving slit, 0.3 mm; X-ray source, Cu-Kα; Cu filter element, Ni; X-ray wavelength, 0.1541 nm; voltage, 30 kV; current, 15 mA; step interval, 0.2 deg.; measurement range, 5.0 - 45.0 deg. as 2Θ. The lattice constants of ZnClAp were also calculated from the results of PXRD analysis using CellCalc software ver. 2.20 developed by Miura.$^{26}$

ATR-IR spectroscopy
ATR-IR spectra of the synthesized samples were measured with an FT-IR spectrometer (FT-IR 6200, JASCO Co., Tokyo, Japan). Measurement conditions were as follows: prism, diamond; resolution, 4 cm$^{-1}$; cumulated number, 32; measurement range, 600-4000 cm$^{-1}$.

Quantitative analyses
The synthesized samples were respectively dissolved in 0.1 mol/L acetate buffer (pH 4.2) for the subsequent quantifications of calcium, phosphate and chloride ions. Calcium and zinc ions were determined by a chelatometry at pH 10 (NH$_3$-NH$_4$Cl buffer) using 0.001 mol/L ethylenediaminetetraacetic acid
disodium salt and Eriochrome Black T as a titrant and a metal indicator, respectively.

Phosphate ion was determined by a Molybdenum Blue spectrophotometry. Color reagent was prepared by mixing aqueous solutions of 0.408 mol/L L-ascorbic acid and 0.0097 mol/L ammonium molybdate at the volume ratio of 1:5. Standard solution of phosphate ion was prepared by dissolving KH$_2$PO$_4$ in pure water. The standard and sample solutions were respectively mixed with 1.5 mL of the color reagent, and then with pure water to make the final volume of 20.0 mL. After 15 min, the absorbance of the resulting blue color was measured at 880 nm with a UV/Vis spectrophotometer (U-3900H, Hitachi High-Tech Science Co., Tokyo, Japan).

Chloride ion was determined by a mercury thiocyanate method. Standard solution of chloride ion was prepared by dissolving well-dried NaCl in pure water. Standard and sample solutions were respectively mixed with 10.0 mL of a color reagent containing 1.65 mol/L iron(III) nitrate and 0.0126 mol/L mercury(II) thiocyanate, and then with pure water to make the final volume of 13 mL. After 10 min, the absorbance of the resulting orange-red color was measured at 460 nm with the UV/Vis spectrophotometer.

The morphology of the synthesized samples was observed with a scanning electron microscope (SEM; JCM5700, JEOL Co. Ltd., Tokyo, Japan). The accelerating voltage of SEM was set at 15 kV. Magnification was 300 or 3000. Prior to the measurement, the surfaces of the synthesized samples were coated with gold by an ion-coating apparatus (IB-3, Eiko Engineering Co. Ltd., Tokyo, Japan) in order to increase the sensitivity. The coating time was 3 min.

Storage experiments at high humidity

The synthesized samples were stored at 37°C in 100% relative humidity for 7 days. After the storage, the samples were dried in a drying oven (MOV-112, SANYO Electric Co. Ltd., Osaka, Japan) at 37°C for 24 h.

X-Ray computed tomography (X-Ray CT)

X-ray computed tomography (X-ray CT) was measured with a SkyScan 1176 high resolution X-ray micro-CT system (Bruker microCT, Kontich, Belgium). Measurement conditions were as follows: filter setting, no filter; source voltage, 40 kV; source current, 600 μA; pixel size, 35 μm. The closed porosity, predictive weight, volume and density were evaluated using X-ray CT software (CT Analyser, Version: 1.15.4.0, Bruker microCT, Kontich, Belgium).

Principal component analysis (PCA)

PCA was applied to ATR-IR spectra using a chemometric software (The Unscrambler, Ver.10.4, CAMO, Norway) that is based on a Nonlinear Iterative Partial Least Squares (NIPALS) algorithm. The algorithm is one of the well-known algorithm that are employed for finding the eigenvectors. In the present study, 7056 data points in 600 - 4000 cm$^{-1}$ in total were picked up as spectral variables.

RESULTS AND DISCUSSION

PXRD analysis

Figure 1 shows the PXRD patterns of synthesized samples (Products A and B). The patterns of ClAp, HAp, DCPD, CaCl$_2$, CaO and ZnO are also shown as references. The synthesized samples give major peaks at 26.0°, 32.2° and 39.6°, the Mirror indices of which are (0.0.2), (1.1.2) and (3.1.0), respectively. These peaks are characteristic of HAp structure, which shows peaks at 25.8°, 31.8°, 32.2°, 32.8° and 39.8°. It is reported that the HAp peaks slightly shift when divalent ions except Ca$^{2+}$ (i.e., Zn$^{2+}$ in this study) are substituted in HAp. The lattice constants of the products are also affected by divalent ions. In addition, the synthesized samples show no detectable peaks of DCPD, CaCl$_2$, CaO and ZnO. These results indicate, therefore, the starting materials are converted to ZnClAp (Product A) and ZnHAp (Product B) by the present mechanochemical methods.

![Figure 1: PXRD patterns of synthesized samples and reference materials](image)

Products A and B were produced by Synthetic methods 1 and 2, respectively. The water/solid ratio for both the syntheses was 1.0 mL/g.

Lattice constants were calculated from the results of PXRD in the range of 5.0 - 45.0°. The crystal structure of HAp belongs to hexagonal system. The
relationship among the lattice constants \((a, b, c)\) of hexagonal system is \(a = b \neq c^{32}\). The lattice constants of synthesized ZnClAp, ZnHAp, ClAp and HAp are 9.429, 9.252, 9.526 and 9.410 Å in the \(a\) axis and 6.842, 6.850, 6.832 and 6.809 Å in the \(c\) axis, respectively. The \(a\) axis lattice constant of ZnClAp was greater than those of ZnHAp and HAp. This is due to the presence of Cl\(^{-}\) in the ZnClAp crystal. Crystal ionic radius of Cl\(^{-}\) (1.81 Å) is larger than that of OH\(^{-}\) (1.35 Å), also resulting in the increase of the lattice constant as mentioned above. The difference of the \(a\) axis lattice constants between ZnClAp and ClAp (i.e., 9.429 Å and 9.526 Å, respectively) may be explained by the similar reason. That is, crystal ionic radius of Zn\(^{2+}\) (0.74 Å) is smaller than that of Ca\(^{2+}\) (0.99 Å)\(^{33}\).

**ATR-IR spectroscopy**

Figure 2 shows ATR-IR spectra of the synthesized samples and the reference materials. The products show the peaks at 600 and 1021 cm\(^{-1}\) that are typical of HAp structure\(^{34,37}\). The peaks are assigned to \(\nu_3\)PO\(_4\) and \(\nu_2\)PO\(_4\) derived bands, respectively. Broad bands of adsorbed water are also observed in the ranges of 1590 - 1700 cm\(^{-1}\) and 3000 - 3600 cm\(^{-1}\). The water was supplied from the bound water of CaCl\(_2\) (see ATR-IR spectrum of CaCl\(_2\)). In addition, water added with the starting materials is also considered to be the cause. Although the samples were dried at 37°C for 24 h, the adsorbed water was not removed. These results also indicate that ZnClAp (Product A) and ZnHAp (Product B) are successfully produced by the present synthetic methods, although the products contain a small amount of H\(_2\)O. It is reported that H\(_2\)O affects physicochemical properties (crystallinity, solubility, and so on) of HAp-based biomaterials\(^{38,39}\), resulting in the change of their biocompatibilities.

**Composition of synthesized ZnClAp**

Composition of the synthesized ZnClAp, determined by the quantitative analyses, were 4.82, 2.83 and 0.565 as mole fraction for Ca\(^{2+}\) + Zn\(^{2+}\), PO\(_4\)^{3-} and Cl\(^{-}\), respectively. The molar ratio of calcium and zinc to phosphorus and that of calcium and zinc to chlorine are, therefore, 1.70 and 8.54, respectively. These values are deviated from the ideal values of ZnClAp (i.e., 1.67 and 5.00, respectively). The replacement of phosphate ion in ZnClAp by the formation of HAp as a by-product are considered to be responsible for the respective deviations. Assuming that only ZnHAp and HAp are by-products, the synthesized ZnClAp contains 43.5% of by-products in molar basis.

**Storage experiments**

**PXRD analysis**

Figure 4 shows the PXRD intensities at 32.2° (2\(\theta\)) for the synthesized ZnClAp samples before and after the storage experiment (37°C, 100% relative humidity, 7 days). The intensities for CIAp (i) and HAp (j) that were dried at 37°C and had not been subjected to high humidity are also shown as references. Although the crystallinities of the samples were retained after the storage in high humidity, they were lower than those of CIAp and HAp. It is reported that the divalent cations such as Zn\(^{2+}\) suppress the crystal growth of HAp\(^{40}\). The low crystallinities of the synthesized ZnClAp is, therefore, attributed to the suppression of crystal growth by Zn\(^{2+}\).
FIGURE 4  PXRD intensities ($2\theta = 32.2^\circ$) patterns of the ZnClAp (a-h), ClAp (i) and HAp (j). Blue bars (a, c, e, g) and red bars (b, d, f, h) respectively indicate the intensities before and after the storage experiment. Water/solid ratio for the mechanochemical synthesis: 1.2 (a, b), 1.0 (c, d), 0.75 (e, f), 0.5 mL/g (g, h).

ATR-IR spectroscopy

Figure 5 shows ATR-IR spectra of ZnClAp before and after the storage experiment. Contrary to the PXRD results, ATR-IR results showed a slight change in the crystallinity during the storage in high humidity. That is, IR band for $\nu_3PO_4$ at 1021 cm$^{-1}$ decreased during the storage, meaning that the crystallinity of ZnClAp decreased. The IR bands for adsorbed water at 1650 and 3300 cm$^{-1}$ also decreased after the storage. This result indicates that the adsorbed water was released from ZnClAp during the storage in high humidity.

FIGURE 5  ATR-IR spectra of ZnClAp before (b, d) and after (a, c) the storage experiment at 37°C. Water/Solid ratio for the mechanochemical synthesis: 1.2 (a, b), 0.5 mL/g (c, d).

Scanning electron microscope

Figure 6 shows the SEM images of synthesized ZnClAp (a,b) and ClAp (c). The surface of ZnClAp seems rough (a,b) in contrast with that of ClAp (c). As mentioned above (PXRD analysis in Results and discussion section), ion radius of Zn$^{2+}$ is smaller than that of Ca$^{2+}$. In addition to the crystal growth suppression by Zn$^{2+}$, discussed in the preceding section, the difference in crystal ionic radius between Zn$^{2+}$ and Ca$^{2+}$ may be attributed to the roughness of ZnClAp surface.

FIGURE 6  SEM images of the synthesized ZnClAp (a, b), ClAp (c) Water/solid ratio for the mechanochemical synthesis: 1.0 (a), 0.5 mL/g.

X-Ray computed tomography (CT) scan

X-Ray CT images

Figure 7 shows the X-Ray CT images of the synthesized ZnClAp samples after the storage experiment in high humidity. There were no noticeable cracks in the interior. In the images, the white part corresponds to the region where the density of the sample is high. It shows that the density at the surface is higher than that in the interior. This result indicates that the crystallinity of the mechanochemical synthesized ZnClAp is higher at the surface than in the interior.

FIGURE 7  X-Ray CT images of the synthesized ZnClAp samples after the storage experiments. Water/Solid ratio for the synthesis: 1.2 (a), 1.0 (b), 0.75 (c), 0.5 mL/g (d).
Figure 8 shows the volume (a), weight (b), density (c) and closed porosity (d) of the ZnClAp samples after the storage experiment. All the values decreased with the increase of water/solid ratio. The lower is the density of HAp, the more soluble is the HAp \(^{31}\). Although high porosity facilitates bone replacement, it reduces the mechanical strength of HAp \(^{32}\). The results shown in Fig.7 suggest that ZnClAp with desired properties for biomaterials can be synthesized by changing the water/solid ratio.

**Principal component analysis (PCA)**

The IR spectra of the ZnClAp samples were analyzed in detail by PCA \(^{33,34}\). Cumulative explained variances obtained by the analysis are listed in Table 1. PC-2 was selected as the principal component number because the Validation increment of cumulative explained variances from PC-2 to PC-3 is as low as 1.83% in contrast with 8.81% from PC-1 to PC-2.

|       | PC-1 | PC-2 | PC-3 |
|-------|------|------|------|
| **Calibration** | 75.26 | 86.58 | 89.22 |
| **Validation** | 76.61 | 85.42 | 87.25 |

Figure 9a shows the Loadings. By referring to the ATR-IR spectra shown in Fig. 4, it is appropriate to consider that PC-2 corresponds to the ATR-IR spectrum of ZnClAp. On the other hand, PC-1 can be correlated to the influence of water/solid ratio on ATR-IR spectra, because only water/solid ratio is changed among the samples. The ratio affects \(H_2O\) peak (1624 and 3000 - 3500 cm\(^{-1}\)) and \(v_3PO_4\) peak (1021 cm\(^{-1}\)). The negative shift of the latter is more significant than the positive shift of the former. This result means that \(v_3PO_4\) peak is most affected by the water/solid ratio among the functional groups in ZnClAp.

Figure 9b shows the relationship between the Scores of PC-2 and PC-1. The samples can be classified into two groups, sample i and others (ii - iv). PC-1 of i is positive, meaning that the water/solid ratio is low. PC-2 of i is negative, meaning that \(v_3PO_4\) peak is reduced. That is, the crystallinity of ZnClAp is low at low water/solid ratio. On the other hand, the PC-1 of ii - iv is negative and their PC-2 shows positive tendency compared with i. This result means that the crystallinity is high at higher water/solid ratio. These results suggest that a certain level of water is needed in order to accelerate the crystal growth of ZnClAp. The PXRD results (section 3.5.1) could not show any correlation between the water/solid ratio and the crystallinity of ZnClAp. On the contrary, PCA can clearly reveal the effect of the water/solid ratio on the crystallinity. Therefore, PCA is considered to be useful to know the necessary sufficient water/solid ratio for obtaining high crystallinity ZnClAp.

**TABLE 1. Cumulative explained variance of PCA on ZnClAp ATR-IR spectra**

|       | PC-1 | PC-2 | PC-3 |
|-------|------|------|------|
| **Calibration** | 75.26 | 86.58 | 89.22 |
| **Validation** | 76.61 | 85.42 | 87.25 |

**CONCLUSION**

Zinc chloroapatite (ZnClAp) was synthesized from CaHPO\(_4\)\(\cdot\)2H\(_2\)O, CaO, ZnO, CaCl\(_2\) and water by a mechanochemical method using a planetary ball mill. The crystallinities of the products were evaluated by a powder X-ray diffractometry (PXRD) and an attenuated total reflection - infrared (ATR-IR) spectrometry. Furthermore, principal component analysis (PCA) was applied to the ATR-IR spectra. It is revealed that ZnClAp, a promising biomaterial having better solubility and bone metabolism ability, can readily synthesized by the present method. The crystallinity of the synthesized ZnClAp can be changed arbitrary by selecting an appropriate water/solid ratio for the synthesis.
