Studies on Change in Solubility over Time of the Bioactive Material Amorphous Calcium Phosphate and Precipitation of Hydroxyapatite

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Abstract: In this study, we immersed amorphous calcium phosphate (ACP) powder in biochemical buffer solutions and performed analysis of its solubility and phase transformation of the precipitate. After preparing ACP powder that contains no impurities, we used 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer, one of the good buffers, as a buffer solution and measured the amount of calcium ions eluted from ACP and other calcium phosphate crystals. ACP was immersed in the buffer solution at 5°C, 20°C, and 37°C, and the amount of eluted calcium ions was measured from 15 min to 24 h thereafter. The precipitated solid phase was analyzed using X-ray diffraction and its morphology was observed using transmission electron microscopy. The precipitation of hydroxyapatite (HAp) was observed after 15 min in HEPES buffer solution. Furthermore, in this experimental group, the precipitates of the sample incubated in HEPES buffer solution at 37°C for 24 h produced the largest HAp crystals. From these results we concluded that ACP immersed in HEPES buffer solution easily releases calcium ions and phosphate ions, and a rapid phase transformation to HAp occurs. Moreover, we assume that, in addition to the thermodynamic effect, the crystal growth of HAp is enhanced by the buffer solution.

Key words: Kinetics, Apatite, Good buffer solution, Amorphous calcium phosphate, Bioactive material

Introduction

Classes of bioactive materials used in dentistry include hydroxyapatite (HAp), tricalcium phosphate (TCP), bioactive glass (BG), and amorphous calcium phosphate (ACP). Since these materials contain the same components and have the same composition as teeth and bones, they are used to actively induce tissue regeneration. Among these bioactive materials, ACP has been reported to be present in biological hard tissues such as bones and teeth, and contains the same components and has the same composition as hydroxyapatite (HAp). Since these materials are used in biological hard tissues such as bones and teeth, and exhibit biological properties such as excellent osteoconductivity, biodegradability, and bioactivity. Owing to these excellent characteristics, ACP is applied in cement fillers, dentifrices, and chewing gums in dentistry. ACP is the first solid phase in the precipitation of calcium phosphate crystals and differs from other calcium phosphate crystals in that it has a short-range order and has no long-range order. Furthermore, the solubility of ACP is higher than that of other crystalline calcium phosphates. Therefore, high solubility can be expected also in saliva and other biological buffer solutions. When considering the clinical use of ACP in the oral cavity, considering its behavior in buffer solutions is necessary.

Regarding the crystalline phase that precipitates from supersaturated solutions of calcium and phosphate ions, 1) formation of ACP from calcium phosphate solution and subsequent conversion via an intermediate product, octacalcium phosphate (OCP) like crystals, to HAp have been assumed. However, recent studies have demonstrated that in some cases 2) ACP generated from calcium phosphate solution precipitates HAp without going through OCP, or 3) HAp precipitates directly from calcium phosphate solution.

However, if ACP is immersed in a buffer solution, calcium and phosphate ions are likely to be eluted, and in this case, HAp may precipitate directly. There is a further possibility of final precipitation of HAp from the immersed ACP via either 1) HAp through OCP as described above, or 2) HAp without going through OCP. Moreover, the effect of various proteins and ions have been elucidated on the precipitation and subsequent crystal growth of HAp, so that amelogenin and fluorides, as well as aspartic acid promoted, whereas carbonates and magnesium delayed the action.

As mentioned above, the solubility of ACP is higher compared with other crystalline calcium phosphates, and the dissolved calcium and phosphate ions can work as an effective source of ions for hard tissues. Therefore, when ACP is intended to be clinically applied as a bioactive material, it is often processed as a composite material with proteins and ions to make ACP to release large amounts of ions continuously.

However, if ACP containing no impurities could be applied to dentin as a stable powder, instead of the abovementioned composite material, its applicability as a bioactive material could be expanded. Thus, in this study, we prepared ACP powder without impurities using a biochemical buffer solution, and analyzed its behavior over time. Further, the precipitated solid phase was analyzed using X-ray diffraction (XRD) and subjected to morphological observation using transmission electron microscopy (TEM).
Materials and Methods

Preparation of ACP

ACP was synthesized following the method of Layrolle et al.\textsuperscript{20}. As a preliminary preparation, 75 g of molecular sieves 3A 1/8 (FUJIFILM Wako Chem. Ltd., Tokyo, Japan) were added to 1,500 ml of 99.5% ethanol (Japan Alcohol Trading Co., Ltd., Tokyo, Japan) to prepare anhydrous ethanol. First, 6.88 g of 95% calcium shots (FUJIFILM Wako Chem. Ltd., Tokyo, Japan) were added to 1,000 ml of anhydrous ethanol and heated at 80°C for 4 h through stirring under a nitrogen atmosphere to prepare a solution of calcium diethoxide (Ca(OEt)\textsubscript{2}). Next, a phosphoric acid solution was prepared by adding 200 ml of anhydrous ethanol to 11.67 g of 85% H\textsubscript{3}PO\textsubscript{4} (FUJIFILM Wako Chem. Ltd., Tokyo, Japan), and the phosphoric acid solution was slowly added dropwise to the calcium diethoxide solution while agitated at 10°C or less. After the dropwise addition was completed, the mixed solution was aged for 24 h under a nitrogen atmosphere via stirring at room temperature. Finally, after centrifugation of the precipitate, a powder was obtained after drying in an incubator at 60°C in air for 15 h and in a furnace at 100°C for 5 h.

The synthesized products were analyzed using X-ray diffraction (XRD, RINT Ultima+, Rigaku Ltd., Tokyo, Japan), and broad peaks were observed (Fig. 1A). Spherical particles were observed using transmission electron microscopy (TEM, JEM 1400, JEOL Ltd., Tokyo, Japan), and broad peaks were observed (Fig. 1A). The Ca/P ratio of the obtained ACP was 1.73 ± 0.15.

Selection of buffer solution

The following three biochemical buffers (Table 1)\textsuperscript{21-23} were selected as candidates for the buffer solution to be used in this experiment as these have high buffering capacity in the neutral region, reduced complexation ability with metal ions, and are not susceptible to change in pH depending on the temperature. N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, DOJINDO Lab., Kumamoto, Japan) buffer solution, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Nacalai tesque Ltd., Kyoto, Japan) buffer solution and Tris(hydroxymethyl)aminomethane (Tris, BIO RAD Lab., Tokyo, Japan) buffer solution.

We examined if there was any difference in the amount of calcium ions eluted from ACP using the three buffer solutions (pH 7.4), and found no substantial difference. However, because HEPES buffer solution is characterized by low cytotoxicity\textsuperscript{21} and comparatively small change in pH depending on temperature\textsuperscript{20,22}, in this study we decided to use HEPES in the experiments where temperature was a parameter. Further, the concentration of HEPES buffer solution was chosen to be 100 mmol/l because the amount of calcium ions eluted from ACP reached a plateau at 100 mmol/l.

Determination of the amount of calcium ions eluted from various types of calcium phosphate

The amount of calcium ions eluted from calcium phosphate was...
tested using HEPES buffer solution. The following six types of calcium phosphate were used: ACP, tetracalcium phosphate (TTCP, FUJI FILM Wako Chem. Ltd., Tokyo, Japan), β-tricalcium phosphate (β-TCP, FUJI FILM Wako Chem. Ltd., Tokyo, Japan), α-tricalcium phosphate (α-TCP, FUJI FILM Wako Chem. Ltd., Tokyo, Japan), dicalcium phosphate (DCPA, Alfa Aesar Ltd., Massachusetts, USA) and hydroxyapatite (HAp, FUJI FILM Wako Chem. Ltd., Tokyo, Japan).

In 5 ml buffer solution (pH 7.4) 5 mg ACP was immersed and stirred for 1 min. Then, the mixture was centrifuged and the calcium ions amount in the supernatant was determined by colorimetry (Calci-E-Test Wako, FUJI FILM Wako Chem. Ltd., Tokyo, Japan), measuring the absorbance (UV-1200, Shimadzu Co., Kyoto, Japan) at a wavelength of 610 nm.

Comparison of three types of buffer solutions and deionized water

We tested if there was any difference between the three buffer solutions and distilled and deionized water (DW) in the amount of calcium ions eluted from ACP. The same method was adopted as in the previous experiment, and the buffer solutions used (pH 7.4) were TES buffer, HEPES buffer, and Tris buffer.

Change over time in the amount of calcium ions eluted from ACP at different temperatures

In this study, preliminary experiments were conducted to measure the amount of calcium ions elution from ACP in HEPES buffer or DW, respectively. As a result, a significant difference was observed between HEPES and DW in the value of calcium ions elution amount. In addition, the calcium ions elution amount was compared and observed by changing the temperature of the buffer solution and the incubation time of ACP. As a result, the amount of calcium ions eluted in the HEPES solution at 5°C continued to increase until 6 h later, while the amount of calcium eluted in the HEPES solution at 37°C reached a peak within 15 min after the start of incubation, and then gradual decrease was observed. For the purpose of investigating changes in ACP in more detail with reference to these preliminary experiments, the temperature of the experimental conditions was set to 5°C, which is close to the refrigeration temperature, 20°C as the room temperature, and 37°C, which is close to the body temperature. The tendency of increase / decrease in the amount of calcium ions elution with the passage of time was set after 15 min, 1, 2, 3, 4, 5, 6, and 24 h.

In 500 ml HEPES buffer (pH 7.4) 250 mg ACP was immersed and agitated for 1 min, and then 500 μL was collected. The mixture was then centrifuged and the calcium ions content in the supernatant was measured. The samples were kept at each temperature until the end of the experiment. The control for HEPES buffer solution was DW. The abbreviations of the samples are shown in the Table 2.

XRD analysis

The analysis of the samples of 5HE, 5DW, 37HE and 37DW after 15 min and 24 h was performed using XRD with CuKα radiation (λ = 1.5418Å) (RINT Ultima+, Rigaku Ltd., Tokyo, Japan) using a Kβ filter and adopting an integrated X-ray powder diffraction software package (PDXL2, Rigaku Ltd., Tokyo, Japan).

The measurement conditions were as follows: tube voltage 40 kV, tube current 30 mA, 3° to 60° (2θ axis), and 0.02 deg step width. The scanning speed was 2 deg/min only at the time of identifying the ACP synthesized, and 10 deg/min for the other samples.

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Table 2. Abbreviations of the experimental samples

| Temperature | Buffer Solution | 15 minutes | 24 hours |
|-------------|-----------------|------------|----------|
| 5°C         | HEPES           | 5HE        | 5HE-15m  | 5HE-24h  |
|             | DW              | 5DW        | 5DW-15m  | 5DW-24h  |
| 20°C        | HEPES           | 20HE       | 20HE-15m | 20HE-24h |
|             | DW              | 20DW       | 20DW-15m | 20DW-24h |
| 37°C        | HEPES           | 37HE       | 37HE-15m | 37HE-24h |
|             | DW              | 37DW       | 37DW-15m | 37DW-24h |

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Figure 2. Results of measuring the calcium ions amount eluted from various calcium phosphates. The values for ACP and TTCP were significantly higher than those for β-TCP, α-TCP, DCPA, and HAp. The values were expressed as mean ±SD, n=7, *p<0.05.

Figure 3. Amount of calcium ions eluted from ACP in the three buffer solutions. The three buffer solutions showed no significant difference. The values are expressed as mean ±SD, n=7, *p<0.05.
Figure 4. A: Measured changes over time in the amount of eluted calcium ions from ACP at various temperatures. 37HE decreased between 15 min and 24 h. The values was expressed as mean ±SD (n=3). B: Eluted calcium ions amount per unit time from 15 min to 5 h. 37HE showed a negative value.

Figure 5. XRD patterns of ACP immersed in buffer solution and DW at 5°C after 15 min and 24 h. Peaks (▼) corresponding to the diffraction angles of HAp were detected in the samples immersed in HEPES (A, B). A: 5HE-15m. B: 5HE-24h. C: 5DW-15m. D: 5DW-24h.

Figure 6. XRD patterns of ACP immersed in buffer solution and DW at 37°C after 15 min and 24 h. Peaks (▼) corresponding to the diffraction angles of HAp were detected in the samples immersed in HEPES (E, F). E: 37HE-15m. F: 37HE-24h. G: 37DW-15m. H: 37DW-24h.
Transmission electron microscopy

TEM (JEM 1400, JEOL Ltd., Tokyo, Japan) was used to observe the samples 5HE, 5DW, 37HE, and 37DW after 15 min and 24 h. In 2 ml anhydrous ethanol, 0.5 mg of the dried sample powder was suspended and the suspension was dropped onto a collodion membrane. The acceleration voltage was 80 kV and the samples were unstained.

Statistical analysis

The experiment was conducted 7 times and analyzed by Kruskal-Wallis test and Steel-Dwass test. Data were presented as the mean±SD. Statistical analyses were performed using JMP (Version 16, SAS Institute Inc, USA) at a significance level of 0.05.

Results

Determination of calcium ions amount eluted from various calcium phosphates

The amounts of calcium ions eluted were 9.57 mg/dl for ACP, 7.78 mg/dl for TTCP, 1.52 mg/dl for β-TCP, 1.16 mg/dl for α-TCP, 1.11 mg/dl for DCPA, and 0.64 mg/dl for HAp. The values for ACP and TTCP were significantly higher than those for β-TCP, α-TCP, DCPA, and HAp (p<0.05), but no substantial difference between ACP and TTCP was observed (Fig. 2).

Comparison of the three buffers and DW

We found a significant difference (p<0.05) in the amount of eluted calcium ions between the three buffers (TES buffer, HEPES buffer, and Tris buffer) and DW. However, no significant difference was observed among the three buffer solutions (Fig. 3).

Change over time in the amount of calcium ions eluted from ACP at different temperatures

The amount of eluted calcium ions was measured from 15 min to 24 h at 5°C, 20°C, and 37°C. For 5HE, the amount of calcium ions increased from 5.92 mg/dl to 10.21 mg/dl between 15 min and 3 h. Thereafter, no change was observed in the amount until 24 h later. For 20HE, the amount increased from 6.14 mg/dl to 7.37 mg/dl between 15 min and 3 h. Thereafter, it decreased to 6.37 mg/dl until 24 h later. In the case of 37HE, the amount of eluted calcium ions decreased from 6.61 mg/dl to 4.17 mg/dl between 15 min and 24 h. For DW, the amount of eluted calcium ions tended to be lower than that for HEPES at each temperature, and no specific trend owing to temperature difference was observed (Fig. 4A).

The amount of calcium ions eluted per unit time from 15 min to 5 h was positive in terms of 0.91 (mg/dl)/H for 5HE and 0.1 (mg/dl)/H for 20HE, while negative at −0.20 (mg/dl)/H for 37HE. The values for 5DW, 20DW and 37DW were 0.16 (mg/dl)/H, 0.11 (mg/dl)/H and 0.13 (mg/dl)/H, respectively (Fig. 4B).

X-ray diffraction analysis

After 15 min of immersion of ACP in HEPES at 5°C, the presence of OCP in the samples could not be completely denied, but its appearance was unlikely (Fig. 5A). After 24 h, peaks consistent with the diffraction angles of HAp were detected (Fig. 5B). Meanwhile, for the sample immersed in DW at 5°C, although a peak consistent with α-TCP appeared temporarily after 15 min (Fig. 5C), the peak disappeared after 24 h, and a broad peak characteristic of ACP was observed approximately 30° (Fig. 5D).

In the ACP sample immersed in HEPES at 37°C, peaks consistent with the diffraction angles of HAp were detected after 15 min and 24 h (Fig. 6E, F). However, for the sample immersed in DW at 37°C, a peak consistent with α-TCP appeared temporarily after 15 min (Fig. 6G), and peaks consistent with the diffraction angles of HAp were detected after 24 h (Fig. 6H).

Transmission electron microscopy

In the sample of ACP immersed in buffer solution at 5°C an accumulation of fine grained precipitate was shown after 15 min (Fig. 7A). After 24 h, transformation into comparatively large grained crystal like precipitate was observed (Fig. 7B). In the case of the sample immersed in DW at 5°C, spherical particles characteristic of the ACP morphology were observed equally after 15 min and 24 h, and no substantial change was observed (Fig. 7C, D). However, regarding the sample of ACP immersed in HEPES at 37°C, small thin granular or plate like precipitates were observed after 15 min (Fig. 7E). However, after 24 h, plate-like crystals with considerable growth in the c-axis direction were observed (Fig. 7F). The thickness (a and b axes) of these plate like crystals was 5.63 nm ± 0.28 (n=10). For the sample immersed in DW at 37°C, spherical...
particles with characteristic ACP morphology were observed after 15 min (Fig. 7G), and immature small oval precipitates were seen after 24 h (Fig. 7H). No spherical particles characteristic of the ACP morphology were observed in the sample immersed in HEPES at 5°C or 37°C.

**Discussion**

In this study, we immersed ACP powder in a biochemical buffer solution to analyze its solubility and the phase transformation of the precipitate. Therfore, a “good buffer” was used as buffer solution. Among the features of a “good buffer”, the ones most important for this experiment are as follows: good solubility in water and possibility to prepare concentrated buffer solutions; an acid dissociation equilibrium hardly affected by concentration, temperature, and ionic composition; reduced complexation ability with metal ions; and easy detection of target components owing to lack of absorption in the visible and UV range20).

Using HEPES buffer solution, one of these good buffers, Eanes et al. reported that at first ACP precipitates from calcium phosphate solution that is followed by precipitation of HAp via OCP like crystals21). Furthermore, the studies of He et al. in 2020 regarding the precipitation of HAp from calcium phosphate solution using HEPES buffer solution confirmed a passway via precipitation of HAp through ACP, and another involving the direct precipitation of HAp without ACP intermediates22).

Thus, there is a passway in which ACP undergoes phase transformation to HAp via OCP like intermediate product (Passway 1)23), a route in which the phase transformation of ACP to HAp occurs without intermediate products (Passway 2)24), and the one in which calcium and phosphate ions form clusters to directly precipitate HAp (Passway 3)25).

Passway 1 includes two processes, in which the phase transformation can be described as follows:

- From ACP to OCP by
  \[ \text{Ca}_9(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O} \rightarrow \text{CaH}_2(\text{PO}_4)_2 \cdot 5\text{H}_2\text{O} + \text{Ca}^{2+} + 2\text{OH}^- \]

- From OCP to HAp by
  \[ \text{CaH}_2(\text{PO}_4)_2 \cdot 5\text{H}_2\text{O} + \text{Ca}^{2+} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 4\text{H}^+ \]

Then, in the phase transformation of Passway 2, the process from ACP to HAp is expressed as

\[ \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + \text{Ca}^{2+} + 2\text{OH}^- \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \]

Furthermore, in the Passway 3 the phase transformation from monomers to HAp is expressed as

\[ \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + \text{Ca}^{2+} + 2\text{OH}^- \rightarrow \text{Ca}_{14}(\text{PO}_4)_6(\text{OH})_2 \]

Here,

- ACP: \( \text{Ca}_9(\text{PO}_4)_6 \)
- OCP: \( \text{CaH}_2(\text{PO}_4)_2 \cdot 5\text{H}_2\text{O} \)
- HAp: \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \)
- Nano Sized Tri-Calcium phosphate complex: \( \text{Ca}_{14}(\text{PO}_4)_6 \)

In this study, the appearance of OCP peaks could not be confirmed by XRD in any samples after 15 min in the experimental groups. Therefore, the rapid phase transformation of ACP is likely to occur owing to the quick exchange of ions in Passways 1, 2, and 3 by HEPES buffer solution. However, because phase transformation to HAp via OCP has been reported, the possibility of OCP appearing during this 15 min period cannot be excluded. In the future, thoroughly investigating the changes of the crystal phase within an even shorter time is necessary.

The question arose whether the rapid phase transformation of ACP described above depends on the temperature of the solution. According to the results of this study, the amount of calcium ions eluted from ACP increased with time at a solution temperature of 5°C, but conversely, the amount decreased with the passage of time at 37°C. The plausible reason for the decrease in eluted calcium ions amount at 37°C may be that the eluted calcium ions precipitates as a crystalline substance rather than an amorphous one. To confirm the appearance of the crystalline phase, the samples were analyzed using XRD over time, and at 37°C, the peaks matching the diffraction angles of HAp appeared with the passage of time. It was therefore confirmed that HAp precipitates either from the eluted calcium ions and phosphate ions or from ACP.

We used ACP containing no impurities in this experiment but it has been reported26) that this ACP has higher solubility than the crystalline matter. Our study also found that ACP has significantly higher solubility than other crystalline materials. In addition, the ionic strength of the buffer solution was higher compared with that of the DW solution, and because of overwhelming presence of anions and cations, we assumed that calcium ions and phosphate ions can be easily eluted. This suggests that a large number of ions are eluted when ACP is immersed in the HEPES buffer solution, and that HAp is directly precipitated from the eluted calcium and phosphate ions, and simultaneously, HAp is precipitated from ACP via phase transformation.

Results of analyzing the XRD peaks showed that at both 5°C and 37°C, the samples immersed in HEPES underwent phase transformation into HAp after 15 min, but those immersed in DW were not transformed into HAp after 15 min. From these results we concluded that the buffering capacity of HEPES has more impact on phase transformation than the temperature of the solution.

At higher temperature of the buffer solution, crystalline HAp precipitated in a short time, and ACP incubated in HEPES buffer solution at 37°C for 24 h produced the largest HAp crystals in this experimental group. Heat and the buffering capacity of HEPES are the two possible factors for crystal growth. Crystal growth is accelerated by heat, but the buffering capacity of HEPES is also has an effect. HAp can exist as a stable phase in a neutral environment of pH 7 compared with OCP, DCPD, and TCP, and maintaining the pH of HEPES at 7.4 in this experiment can explain the crystal growth of HAp27).

In this study, it was found that ACP immersed in HEPES buffer easily releases calcium and phosphate ions and does not form a complex with HEPES, the phase transformation into HAp proceeds promptly owing to the quick ion exchange, and HEPES buffer solution efficiently enhances HAp crystal growth. These results suggest that the combined use of HEPES buffer and ACP may be effective for HAp precipitation.

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**Conflicts of Interest**

The authors have declared that no conflicts of interest exist.
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