Efficient zinc incorporation into hydroxyapatite through crystallization of an amorphous phase could extend the properties of zinc apatites

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Abstract
Zinc offers many benefits for bone growth and an antimicrobial action for implants. Nearly all studies have synthesized crystals in solution by precipitation, but this produces low crystallinity and poor thermal stability. This work addresses a new approach of incorporating zinc in an amorphous calcium phosphate followed by crystallization at an elevated temperature. A 10 mol% Zn substitution in an amorphous phase only produces 3 mol% incorporation of calcium sites due to zinc removal in an ammonia complex, but 14 mol% Zn inclusion is achieved with hydrothermal processing of an amorphous calcium phosphate in zinc nitrate. Thermal analysis of a 5 mol% Zn enriched apatite tested up to 1000 ^{o}C produces a thermally stable apatite, unlike apatites produced by precipitation. This new method provides more complete Zn filling of Ca (II) sites, is more suitable for thermal processing and potentially extends the performance of Zn enriched apatites for use in dentistry and orthopaedics. This also provides direct evidence about crystallization of zinc enriched amorphous calcium phosphate showing that zinc stabilizes the amorphous phase leading to higher crystallization temperatures.

Keywords: zinc, hydroxyapatite, structure, amorphous calcium phosphate, antimicrobial resistance

Introduction
Zinc promotes bone growth [1], inhibits bone resorption [2], offers antimicrobial resistance [3], and unmatched potential for imparting multi-functionality in bone implants. The availability of zinc determines the performance and this can be controlled by the microstructure (crystal size, shape, crystallinity, zinc content). Calcium phosphates have numerous substitutions and defect states and allow the release of zinc to be tuned. This work will show a new route for producing a thermally stable zinc enriched apatite.

Zinc concentrations will vary for different functions. In-vitro cytotoxicity of osteoblasts has been shown for ZnO at 1.2 wt.% [4], but animal studies indicate that 0.3 wt.% is favourable for bone growth, and even this level can lead to bone resorption after 60 weeks [5]. The best zinc concentration and the release profile need to be designed for multifunctionality, but this requires careful implant design, and a choice of processing technologies to impart a range of properties.

Most studies incorporate zinc from an aqueous solution during crystallization of apatite and often report many complications. Zinc significantly decreases the crystal growth rate by adsorbing to the surface [6] giving a three to four-fold reduction in crystallite size [7, 8], as well as a decrease in structural order, as observed from the X-ray diffraction peak broadening [9]. While maintaining a nanosize may be advantageous, it is not clear whether zinc is in a crystalline or amorphous phase.

Replacement of Ca with the smaller Zn distorts the lattice and introduces a Ca vacancy to lower the (Ca+Zn)/P molar ratio [9]. Crystallization of hydroxyapatite in an aqueous solution includes Zn in the Ca(II) site, next to the hydroxyl column, together with a Ca vacancy [10], Figure 1. Up to 1.5 to 2 atoms can be substituted for calcium in $Ca_{10}(PO_4)_6(OH)_2$.

An interstitial site for Zn is also possible in apatite. Heating a sol-gel synthesized Zn enriched hydroxyapatite (above 900 ^{o}C) causes dehydroxylation to form O-Zn-O chains in the column, in place of OH-OH [11, 12]. This allows one additional zinc atom in the formula unit to produce $Ca_{10}Zn(PO_4)_6O$. Insertion of Zn within the hydroxyl column is the most energetically favourable, according to density functional theory calculations, followed by the Ca (II) and then the Ca (I) site [13]. Processing plays a decisive role in Zn location.

This work will investigate crystallization of zinc substituted amorphous calcium phosphate at an elevated temperature or a combination of temperature and pressure. Thermal crystallization, heating an amorphous phase in a furnace, has not

been investigated for smaller cations such as zinc, but has shown ease of calcium replacement to form strontium substituted hydroxyapatite [14].

Amorphous calcium phosphates containing zinc have not received any attention other than the explanation of their possible appearance with crystallized apatites. This is related to the ability of zinc ions to inhibit the growth of hydroxyapatite crystals [15] and to lower the degree of crystallinity. Gomes et al has referred to the possible crystallization at 500-700 ^{o}C, but did not provide any direct evidence [11].

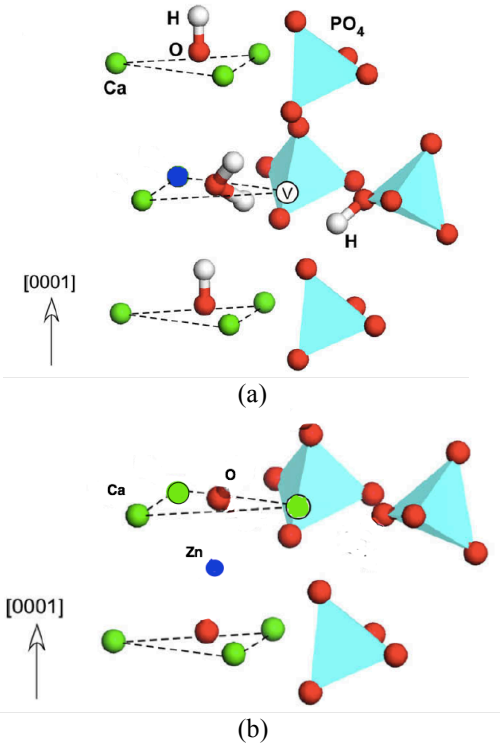


Fig. 1: Position of zinc a) replacing calcium in a complex with a calcium vacancy and two protons and b) as an interstitial site within the column. Zn is in blue, Ca in green. Fig 1 is modified from [10], and Fig 1b is graphically interpreted from information in [11, 12].

The objective is to a) investigate zinc inclusion in an amorphous calcium phosphate, b) determine the crystallization temperature, and c) show whether an apatite structure can be retained after thermal and hydrothermal crystallization of an amorphous calcium phosphate.

Methods

Synthesis of Zn enriched apatite

Synthesis of a zinc enriched apatite was investigated by inclusion in an amorphous phase followed by crystallization, or by hydrothermal treatment in zinc nitrate. The reactants included $Ca(NO_3)_2{\cdot}4H_2O$, $(NH_4)_2HPO_4$, $(NH_4)_2CO_3$, a 33% ammonia solution and $Zn(NO_3)_2{\cdot}6H_2O$. Deionized water was used throughout all the experiments. Pure and substituted HAp with various Zn concentrations (10 and 20 mol%) were prepared.

Precipitation method. To synthesize pure HAp, an aqueous solution was prepared by dissolving $Ca(NO_3)_2{\cdot}4H_2O$ into 250 mL deionized water. A phosphate solution was prepared by dissolving $(NH_4)_2HPO_4$ and $(NH_4)_2CO_3$ into 125 mL deionized water. The $(NH_4)_2HPO_4/(NH_4)_2CO_3$ solution was added to the $Ca(NO_3)_2{\cdot}4H_2O$ solution at room temperature. The pH value was kept above 10 by adding 30 mL ammonium hydroxide. The suspension was stirred, filtered and washed several times with deionized water. The resulting precipitate was freeze dried. Calcination was performed at 700 °C for 15min.
10 and 20 mol% zinc substituted hydroxyapatite were prepared by substituting the calcium nitrate with zinc nitrate.

Hydrothermal method. $Zn(NO_3)_2{\cdot}6H_2O$ was dissolved into 3 mL deionized water and then added to 0,5 g pure amorphous phase or hydroxyapatite. The hydroxyapatite was made by hydrothermal treatment of the amorphous phase at 150 ^{o}C for 4 hours. The powder (ACP or apatite) was placed into the 18ml hydrothermal vessel and heated at 150 °C for 2 hours. The resulting precipitate was filtered and dried at room temperature overnight.

Nitrate solutions provide a better zinc adsorption to apatite surfaces than chloride solutions, and so both calcium and zinc nitrate were chosen as reactants [16].

Characterization

Thermogravimetric and differential thermal analyses (Seiko EXSTAR 6000 TG/DTA 6300) were conducted to determine the crystallization temperature of the Zn enriched amorphous calcium phosphate. Powdered samples were heated to 1100 °C in air at a heating rate of 10 °C/min.
Freeze dried powder with the expected 20 mol% Zn substitution was heated at 700, 800, 900 and 1000 °C for 15 min in a tube furnace to confirm the thermal stability. The samples were then analyzed with XRD.

X-ray powder diffraction (XRD) was used to study the effect of calcination and hydrothermal processing on the final phase. Analysis was carried out with a Bruker D8 ADVANCE diffractometer. Diffraction patterns were recorded from 5° to 60° using Cu Kα radiation (λ = 1.54180Å generated at 40 mA and 40 kV) at a 0.2° step size.

The (Ca+Zn)/P ratio of the samples were determined by *X-ray fluorescence (XRF)* in helium atmosphere, using a Bruker S8 TIGER spectrometer equipped with a Rh source.

Results and Discussion

Zinc inclusion in amorphous calcium phosphate

Zinc transfer from solution into the amorphous calcium phosphate was not efficient. Only 25% of the available zinc was included in the solid regardless of the initial concentration, Table 1. This is due to the formation of a zinc ammonia complex that removes available zinc from the solution. Use of sodium hydroxide to increase the pH avoids the removal of soluble zinc [17].

Table 1. Zn content of precipitated Zn ACPs

Sample	Measured Zn mol%	(Ca+Zn)/P
0% Zn	0	2.1
10% Zn	3	2.1
20% Zn	5	2.1

A similar trend has been observed with crystallization experiments in aqueous solutions. Previous work has shown that NH_4OH for adjusting the pH provides 41% Zn inclusion in solution crystallized apatites, but NaOH increases this to 85% [18]. The lower incorporation has been explained by the formation of $Zn(NH_3)_4^{+}$, removing dissolved Zn from the solution thereby lowering the possible incorporation into apatite.

The included zinc depends on the type of reactants and how the zinc is added. High zinc inclusion has been shown with $Ca(OH)_2$ and H_3PO_4 reactants, instead of $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$ [18]. Similarly, the use of calcium acetate and sodium monohydrogen phosphate also supports effective incorporation of zinc into the structure [9]. A further modification on the first approach has even produced a 5% higher zinc concentration by dissolving zinc oxide into orthophosphoric acid before addition of the calcium [19].

The buffer type in synthesizing calcium phosphates will influence the concentration of dissolved metallic species, but may also affect apatite purity. Potassium hydroxide provides a larger cation than sodium that is incorporated at three times lower concentrations in hydroxyapatite [20], but the affect of sodium and potassium on amorphous calcium phosphates has not been reported.

Zinc inclusion can be 5% higher for solution crystallized apatites [19], but 20% higher with amorphous calcium phosphate [17].

The consistent (Ca+Zn)/P ratio indicates that the zinc is directly substituted for the calcium, Table 1. A higher value is formed due to the inclusion of carbonate in place of phosphate in the lattice.

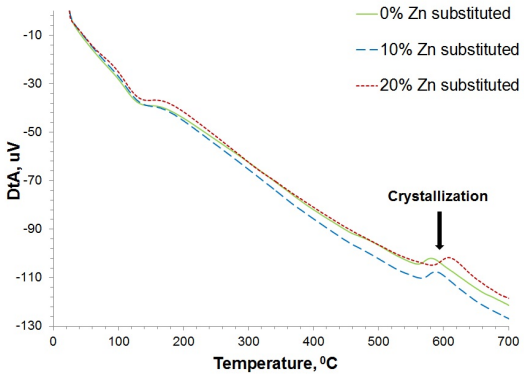
Inclusion of zinc through the amorphous calcium followed by thermal crystallization seems to be easier since a higher defect formation energy is required to incorporate the smaller Zn cation compared to the larger Sr cation [21].

Crystallization of amorphous calcium phosphate

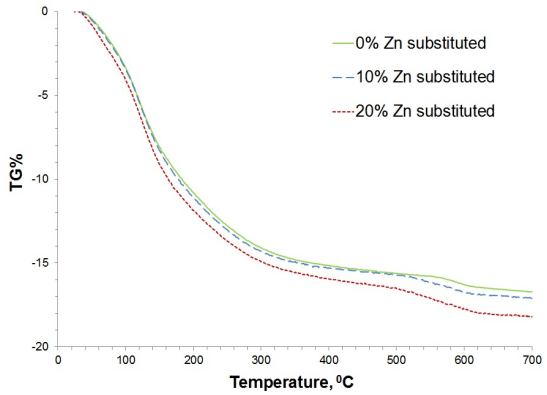
Crystallization requires higher temperatures for greater zinc contents. At 0 mol% Zn the crystallization occurs at 580 ^{o}C, but increases to 585 ^{o}C and further to 607 ^{o}C at higher zinc concentrations, Figure 2a.

Crystallization may be viewed as an indicator of ACP stability. In solution, small cations such as magnesium and zinc have been known to stabilize the amorphous phase and prevent crystallization [22]. Zinc inclusion in carbonated apatites decreases the solubility by about 30% [23]. Despite a slight increase in solubility from carbonate, the small zinc cation bonds strongly to the phosphate to retain the amorphous phase. The higher crystallization temperature for greater zinc contents also suggests stabilization of the amorphous phase.

A small weight loss is observed prior to the crystallization, and this is more noticeable at greater Zn concentrations, Figure 2b.



(a)



(b)

Fig. 2: Thermal analysis showing (a) an increase in crystallization temperature at higher Zn contents (DTA) after b) a small weight loss (TG).

X-ray diffraction shows that heat treatment of the amorphous phase produces crystallization to an apatite structure, Figure 3. Regardless of the zinc content, an apatite is formed.

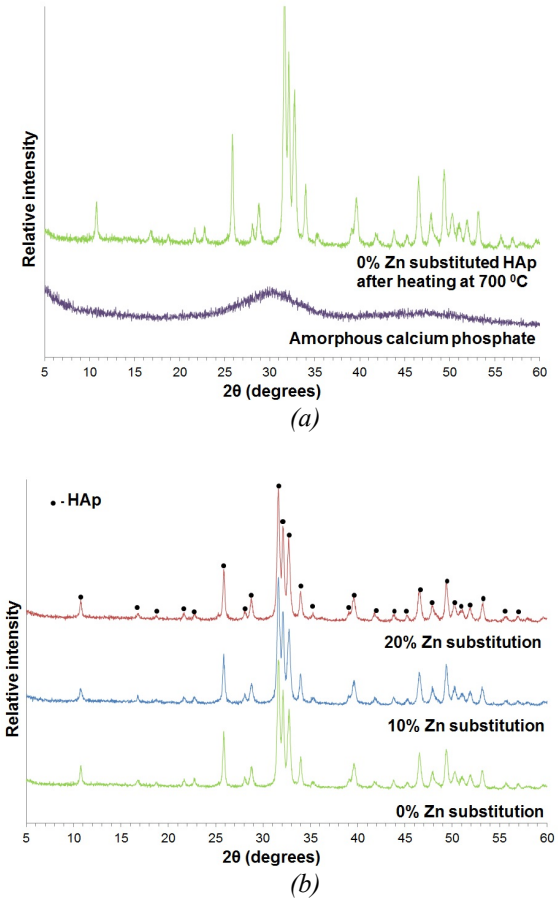


Fig. 3: XRD of a) an amorphous calcium phosphate after synthesis and calcination, and b) calcined amorphous calcium phosphates with different Zn contents. Calcination was made at 700 °C for 15 minutes.

Calcination at higher temperatures still shows phase stability for the crystallized apatites without decomposition, Figure 4. This is consistent with the lack of an exothermic response in the DTA, Figure 2. Others have shown an exothermic response coinciding with decomposition to produce tricalcium phosphate [7].

Unlike the Zn substituted apatite formed during solution crystallization, that introduces a cation defect, thermal crystallization does not appear to include a calcium defect and therefore provides a more thermally stable apatite. This implies that the introduction of calcium defects is characteristic of the crystallization process in aqueous solutions and is not a structural requirement for zinc inclusion.

Up till now, the crystallization of an amorphous calcium phosphate has not been directly shown.

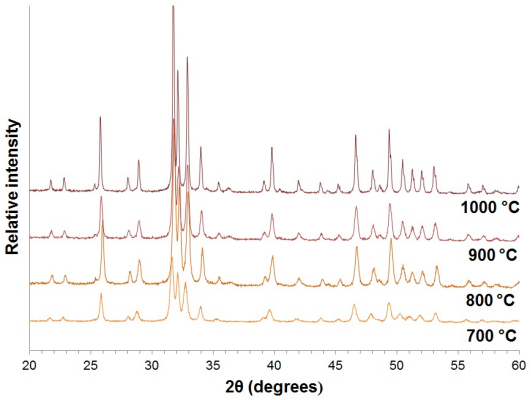


Fig. 4: XRD of 20% Zn amorphous calcium phosphate after heating at 700 °C, 800 °C, 900 °C and 1000 °C for 15 minutes showing a stable apatite at all temperatures.

Calcination of zinc containing apatites has shown new diffraction peaks inferring possible crystallization of an amorphous phase [11]. The crystallization temperatures help differentiate the decomposition of an apatite, containing zinc and calcium vacancy complex, from the crystallization of an amorphous phase.

Hydrothermal processing

Crystallization during hydrothermal synthesis provides the synergy of pressure, temperature and a high vapour pressure to include substitutional cations. An amorphous calcium phosphate was chosen as a lower density structure for possible easier cation inclusion, as compared to apatite.

X-ray fluorescence results suggest that Zn was incorporated at a higher concentration than synthesized amorphous calcium phosphate with zinc, Table 2. A large increase has previously been shown for the amorphous phase, but, according to the known substitution alternatives, it is not expected for crystallized apatites.

Table 2. Zn content of hydrothermally processed calcium phosphates

Sample	Measured Zn mol%	(Ca+Zn)/P
Amorphous calcium phosphate		
0% Zn	0	2.1
10% Zn	14	2.2
20% Zn	27	2.4
Hydroxyapatite		
0% Zn	0	2.1
10% Zn	12	2.1
20% Zn	21	2.1

X-ray diffraction also shows zinc phosphate tetrahydrate (hopeite). Narrow peaks reveal high crystallinity hopeite or large crystals. Hopeite formation is in agreement with other studies that have shown adsorption of zinc onto hydroxyapatite in a pH 3 - 6.5 solution, but zincite (ZnO) at pH 8.5 to 12 [24]. A pH of 3 in the zinc nitrate solution confirmed the conditions suitable for producing hopeite.

Hopeite may form at room temperature conditions [25] and can be confirmed by calcination [26].

Autoclaving has been previously used to increase the crystallinity of solution crystallized hydroxyapatite containing zinc [27]. The hydrothermal treatment of amorphous calcium phosphate in zinc nitrate is more complex.

For the intended 10% Zn substitution into amorphous calcium phosphate, a total of 14% Zn was captured, Table 2. This is close to the limit in the apatite lattice and the broad peaks suggest a lower crystallinity apatite. The low pH of the zinc nitrate supports dissolution of the amorphous calcium phosphate and uptake of zinc to form a low crystallinity apatite. The crystallinity of the hydrothermally crystallized zinc apatite is comparable to a 15 mol% Zn solution crystallized zinc apatite [7, 9].

Adding more zinc in the hydrothermal process leads to a greater zinc content product explained by the appearance of hopeite and calcium zinc phosphate dihydrate (Parascholzite), Figure 5.

Regulation of the pH could avoid the appearance of hopeite and parascholzite, but zincite is preferred at a pH greater than 9 and this has been confirmed by hydrothermal processing of zinc nitrate solutions [28]. A pH greater than 11 will form a zinc ammonia complex and experiments have revealed that an apatite is formed without additional phases. The use of reactants and bases that do not include ammonia show promise for making the zinc available to be incorporated into the amorphous calcium phosphate and further transformed to apatite.

Usually the crystallinity of precipitated crystals is improved by thermal treatment, but the approach used here performed the crystallization at an elevated temperature directly from an amorphous calcium phosphate, either by calcination in a tube furnace at 700 ^{o}C or hydrothermal processing. This has avoided the apparent inclusion of the calcium defect that can lead to decomposition into tricalcium phosphate along with hydroxyapatite. Despite reports of reversion to the apatite structure with further heating [11], the process used here would be more effective in retaining the higher energy from nanoparticles for densification, if a body needs to be produced instead of retaining individual particles.

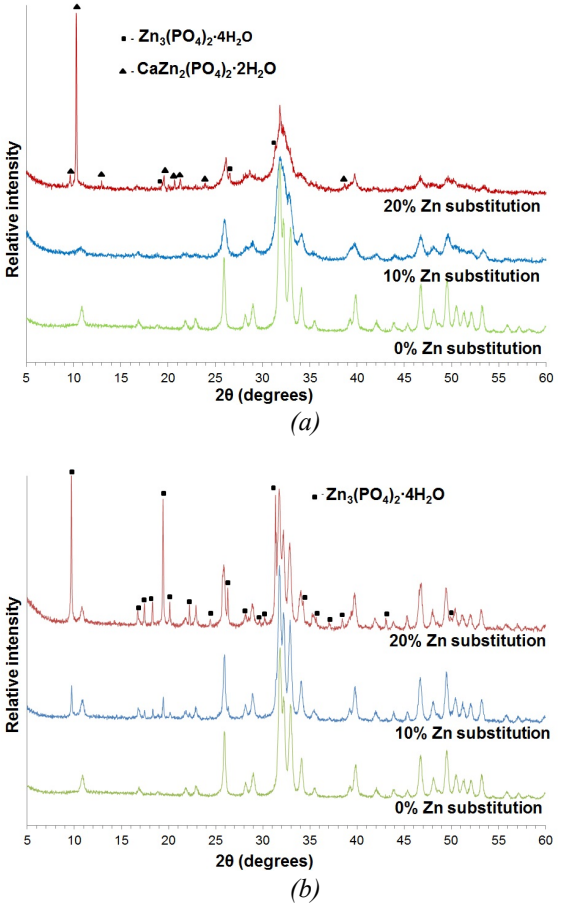


Fig. 5: XRD of hydrothermally processed zinc nitrate with a) ACP and b) HAp. The more soluble ACP provides a calcium-zinc compound, while the less soluble HAp only shows zinc phosphate (hopeite).

The substitution of a calcium with zinc in thermally processed zinc apatites is different from solution crystallized zinc apatites that show a two calcium ion replacement with a zinc ion and a calcium vacancy. This presents a more thermally stable zinc apatite, with a possibly different biological response. Further work could also consider thermally crystallized zinc apatites as an alternative for antimicrobial applications and bone generation.

The amorphous to crystalline transition could also be used for acquiring other zinc containing crystalline phases. This work has yet to be done, but offers great potential. Both zinc containing α-tricalcium phosphate and β tricalcium phosphate are of particular interest for their application in cements, scaffolds and particles.

Conclusions

A new approach for producing zinc apatites was investigated by crystallizing an amorphous calcium phosphate at elevated temperatures or in a

hydrothermal process. Crystallization of a zinc containing amorphous calcium phosphate by calcination provided a more complete zinc incorporation into apatite, as displayed by thermal stability up to 1000 ^{o}C. Zinc incorporation is not efficient in ammonia containing solutions where a zinc ammonia complex removes soluble zinc from solution. Hydrothermal processing achieved zinc incorporation at a 40% higher molar content and produced a 15 mol% zinc incorporation, known as the limit for zinc apatites produced by precipitation.

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