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Amorphous calcium phosphate materials

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Amorphous calcium phosphate (ACP) is essential in formation of mineralized bone and using as a bone substitute. This study presents new aspects of carbonated ACP crystallization during heat treatment. Initially synthesis end pH and drying method (80 °C or freeze-drying) of ACP were varied. Thermal behaviour and structure of differently obtained ACP were evaluated using DSC-TGA, heating microscopy, XRD, FT-IR. In addition, degree of crystallinity (DOC), phase composition and chemical group information were compared for as-synthesized and heat-treated (crystallization end T and 1200 °C) ACP. For the first time DSC-TGA and heating microscopy methods were correlated. DOC of samples dried at 80 °C was synthesis end pH dependent. Heat treatment without temperature hold at crystallization end T produced materials with DOC of 82–91%, thus proving efficiency of low temperature processing. Variations in drying method and synthesis end pH affect structure of the samples heat treated at crystallization end T, but not at 1200 °C.

1. Introduction

Calcium phosphates (CaPs) are of high interest in biomaterial field because of their outstanding response to living tissues and body environment [1]. Human bone is composed of inorganic and organic (collagen and proteins) components [2]. The inorganic part is calcium-deficient, low-crystalline, usually non-stoichiometric and carbonated CaP that highly resembles chemical structure of hydroxyapatite (HAp) [3, 4]. However, high crystallinity and stoichiometry of HAp lead to rather slow rates of dissolution [5] and therefore when used as an implant the process of bone remodelling is slow as well. Amorphous calcium phosphate (ACP) has high solubility, facilitated by its amorphous structure, the hydrated layer and defects. In particular, the lack of periodic long-range order in ACP allows formation of structural defects thus increasing both rates of solubility and resorption leading to improved bioactivity [6]. Use of ACP instead of widely used HAp or biphasic HAp/β-TCP could enhance the bone repair. However, at certain conditions (moisture, different pH and ion environment, elevated temperatures etc.) the metastable ACP transforms into other crystalline CaPs, usually HAp [7], α- or β-tricalcium phosphates [8] or mixtures of these [9]. Despite previous efforts in ACP studies, crystallization of ACP is still not properly understood. Actually presence of ACP in evolving bone was confirmed quite recently in 2008 [10].

Way to understand any amorphous material is to observe heat-induced crystallization of it (formed crystalline phases, associated thermal events etc.) by controlled heat treatments. Such knowledge on heat treated ACP is beneficent in preparation of CaP ceramics, specifically with certain degree of crystallinity (DOC). These CaP materials could mimic not only the chemical composition and chemical properties of bone minerals, but also provide a different starting point for bone repair process in comparison with conventional highly crystalline CaP materials. In fact, DOC is slightly overlooked property, however it influences protein adsorption, cell adhesion and differentiation especially for bone substitutes [11]. There is limited availability on exact DOC of human bone mineral as it is dependent on many factors (human age, bone type, disease history etc.). Newly formed bone usually has smaller DOC than older bone, because the transformation of amorphous phase into the crystalline phase is slow [12]. Grynpas [13] has determined DOC of bone mineral to be 51–58%.

Synthetic CaP with specific DOC can be obtained in several ways: 1) synthesis of CaP by fine tuning of process parameters (T, pH, additives etc.); 2) by aging of CaP suspensions after synthesis (T, time, pressure...
freeze-drying samples were frozen in liquid N2 right after the washing. Dried either in freeze-dryer (72 h) or drying oven (80 °C for 1 h). Prior to separation by centrifuge, washed with deionized H2O (30–40 min) and contact time with water during drying. The overall aim of the study was to evaluate drying methods and synthesis pH impact on carbonated ACP, complemented by analysis of thermal properties and structural features of heat treated products. Further it will give an insight on how to heat treat carbonated ACP to obtain both carbonated and partially crystalline CaP bone graft substitutes.

2. Materials and methods

2.1. Synthesis of amorphous calcium phosphate

ACP was synthesized by re-precipitation from solution containing homogenous mix of calcium and phosphate ions [21]. In brief, from HAp (further designated as R-HAp) initial suspension in water was prepared. R-HAp was dissolved in HCl and later NaOH was added to induce rapid precipitation of ACP. Final pH (8, 9, 10 and 11) of the synthesis was adjusted with diluted NH4OH. Precipitates of ACP were separated by centrifuge, washed with deionized H2O (30–40 min) and dried either in freeze-dryer (72 h) or drying oven (80 °C for 1 h). Prior to freeze-drying samples were frozen in liquid N2 right after the washing procedure. Samples were further abbreviated as FrD or Ov together with corresponding synthesis end pH value, e.g., Ov_pH11.

2.2. Characterisation methodology

2.2.1. Specific surface area and particle size

Specific surface area (SSA) was determined after Brunauer–Emmett–Teller (BET) method using N2 adsorption system Quadrasorb SI (Quantachrome Instruments, USA). Samples were degassed at room temperature for 24 h to remove any moisture and vapours. Particle size d\text{BET} was calculated after equation 1 assuming spherical particle shape [22]:

\[ d_{\text{BET}} = 6/(\rho \times \text{SSA}), \]

where \( \rho \) – density of HAp (2.81 g/cm\(^3\)), determined with Micromeritics AccuPyc 1330.

\[ dBET = 6/(\rho \times \text{SSA}), \]

2.2.2. Fourier transform infrared spectrometry

Chemical groups of samples were characterized with Fourier transform infrared (FT-IR) spectrometer 800 Scimitar Series (Varian, USA) with ATR unit. Scans (n = 50) were acquired in range of 4000-400 cm\(^{-1}\) with resolution of 4 cm\(^{-1}\).

2.2.3. Degree of crystallinity

Degree of crystallinity (DOC) was calculated after at \( T_{\text{cryst.end}} \) + 10 °C and 1200 °C (after full DSC run) to test whether DOC increases by continuing the heat treatment after the detected crystallization event on DSC. The extra 10 °C for \( T_{\text{cryst.end}} \) were added as safety interval to make sure that the end of the crystallization effect was reached. Heating rate was 10 °C/min and samples were left to cool freely. DOC was calculated by dividing integrated intensity of following XRD patterns: sample of interest and the same sample heat-treated at 1000 °C for 15 h [22]. Heat treatment for 15 h would give the most crystalline sample of the same composition.

2.2.4. X-ray diffraction

Powder x-ray diffractometer X’pert Pro (PANalytical, the Netherlands) equipped with Cu tube (Cu Kα = 1.540598 Å) was used for phase determination. Measurements were done in 2θ range of 5-70°, step size was 0.05°, counting time – 69.85 s. Crystalline phases were identified using ICDD PDF-2 database with reference cards 1-072-1243 for HAp, 9-0169 for \( \beta \)-TCP and 9-0348 for \( \alpha \)-TCP. Quantitative amount of crystalline phases was determined using software Maud [23]. Patterns for refinement of HAp [24], \( \beta \)-TCP [25] and \( \alpha \)-TCP [26] were taken from Crystallography Open Database.

2.2.5. Degree of crystallinity

Degree of crystallinity (DOC) was calculated after at \( T_{\text{cryst.end}} \) + 10 °C and 1200 °C (after full DSC run) to test whether DOC increases by continuing the heat treatment after the detected crystallization event on DSC. The extra 10 °C for \( T_{\text{cryst.end}} \) were added as safety interval to make sure that the end of the crystallization effect was reached. Heating rate was 10 °C/min and samples were left to cool freely. DOC was calculated by dividing integrated intensity of following XRD patterns: sample of interest and the same sample heat-treated at 1000 °C for 15 h [22]. Heat treatment for 15 h would give the most crystalline sample of the same composition.

2.2.6. Heating microscopy

Sintering behaviour of as-synthesized samples was observed \textit{in situ} using high temperature microscope (Hesse Instruments, Germany) equipped with Sony B&W camera. Samples were prepared by manual uniaxial pressing into round die thus obtaining cylindrical test piece (\( d = 2.5 \text{ mm}, h = 3.0 \text{ mm} \)). Pressing load of stainless steel punch with integrated spring was approximately 1.5 N/mm\(^2\). The test piece was placed on alumina plate for observation of its cross-section area change. Heat treatment was the same as in DSC runs. Characteristic temperatures were determined as intersection of tangents.
3. Results and discussion

3.1. Characteristics of starting powders

Specific surface area (SSA) and particle size \(d_{\text{BET}}\), XRD patterns and FT-IR spectra of samples are summarized in Table 1, Figs. 2 and 3.

### Table 1

<table>
<thead>
<tr>
<th>Sample abbreviation</th>
<th>Drying method</th>
<th>Specific surface area, (m^2/g)</th>
<th>Particle size (d_{\text{BET}}, \text{nm})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ov_pH8</td>
<td>Oven, 80 °C (Ov)</td>
<td>154 ± 9</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Ov_pH9</td>
<td>[21]</td>
<td>141 ± 8</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Ov_pH10</td>
<td></td>
<td>153 ± 25</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Ov_pH11</td>
<td></td>
<td>150 ± 28</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>FrD_pH8</td>
<td>Freeze drying</td>
<td>115 ± 10</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>FrD_pH9</td>
<td>72 h (FrD)</td>
<td>116 ± 15</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>FrD_pH10</td>
<td></td>
<td>125 ± 16</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>FrD_pH11</td>
<td></td>
<td>120 ± 17</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>R-HAp</td>
<td>Oven, 80 °C (Ov)</td>
<td>95 ± 3</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>n-HAp</td>
<td></td>
<td>12</td>
<td>178</td>
</tr>
</tbody>
</table>

2.2.7. Reference samples

n-HAp nanopowder purchased from Sigma-Aldrich and unsintered R-HAp prepared in the RTU laboratory by wet precipitation method [27] were used as references. n-HAp was chosen as a reference as it represents thermodynamically stable and highly crystalline CaP phase contrary to ACP. R-HAp is also referenced as it was raw material for ACP synthesis and has a structure of partially crystalline CaP.

3.2. Thermal behaviour of differently dried ACP

3.2.1. Thermogravimetric analysis

Curves of thermogravimetric analysis (TGA) are shown on Fig. 4. Mass loss is gradual for both Ov and FrD samples. The adsorbed water is reversibly removed in range from 25 to 200 °C and the chemically bound water is irreversibly lost between 200 and 400 °C [29]. Ammonia releases at temperatures up to 400 °C [30]. Around 550 °C small mass loss step is observed for several samples: Ov_pH9, Ov_pH10 and Ov_pH11.

The mass loss up to 200 °C and the total mass loss at 1200 °C were more expressed for Ov samples. In the case of Ov samples the total mass losses were from 11 up to 20% with increasing value of synthesis end pH. For FrD samples the total mass loss was up to 14%. Mass loss up to 200 °C clearly shows the difference between drying in air and freeze-drying. Larger amounts of physically adsorbed substances remain in the former case. Ways of reducing mass losses when drying in air would be to increase drying time or temperature. However longer drying times [18] and higher drying temperatures [31] lead to crystallization of ACP.

Synthesis pH correlated with the observed mass loss: the higher the pH value, the greater mass losses were observed. This trend was evident both for Ov and FrD samples. The impact of pH on mass losses originates from amount of added ammonia for pH adjustment.

R-HAp and n-HAp have negligible total mass losses – 4.8% and 1.7% at 1200 °C, respectively. TGA analysis confirmed that reference samples are stoichiometric HAp, otherwise a sharp weight loss in 700–800 °C region and a smaller weight loss above 900 °C would be observed [32].

3.2.2. Differential scanning calorimetry

DSC curves present exothermic crystallization effects of ACP phase transforming into crystalline CaP (several phases possible, see Table 2) for each sample (see Fig. 5). Here crystallization is not associated with simultaneous mass loss when compared with TGA curves (see Fig. 4). The negligible mass loss around 550 °C for few samples is completed before start of the crystallization. Reference HAp samples show no thermal effects, as they are composed of the most thermodynamically stable CaP [33].

Characteristic temperatures \(T_{\text{cryst.onset}}\) and \(T_{\text{cryst.end}}\) and enthalpies are depicted on Fig. 6. There is no direct correlation between DSC peak parameters and synthesis end pH, drying method or amount of lost mass in TGA. \(T_{\text{cryst.end}}\) and enthalpy values are slightly higher for the FrD samples (Fig. 6). This is related to structural differences, we could, say, that FrD samples have more distorted structure than Ov samples, therefore FrD samples require more energy to crystallize. However, XRD shows the same pattern for all ACP samples and such intimate differences are not distinguishable.

Particularly, Ov_pH8 has rather big peak area and a wide crystallization temperature region from 623 to 887 °C. It might be a sum of several thermal effects – conversion from already partially crystalline...
CaP with DOC of 50% to crystalline CaP with possible transformation from α-TCP (undetected) to β-TCP. However, for Ov pH8 only β-TCP was found to be present at T\text{cryst.end} and 1200 °C (see Table 2).

The observed crystallization at 630–720 °C in Fig. 5 corresponds to formation of various CaP phases (see Table 2) from ACP. The dominant phase being β-TCP in most studied cases. Somrani et al [34] observed crystallization of ACP at 625 °C (peak temperature) into α-TCP and later to β-TCP. Feng and Khor [35] got exothermic peak with onset of 630 °C for plasma sprayed HAp containing amorphous phase. In their case ACP transformed into mixture of HAp, tetracalcium phosphate and CaO.

Crystallization observed in DSC starts at 150–200 °C higher temperature from the point in TGA where the greatest mass loss (up to
400 °C) was observed. Loss of chemically bound water does not trigger instant crystallization of ACP. Actually, Somrani et al. [34] have observed that water molecules do not directly interact with phosphate groups and do not alter the structure when they leave the ACP on heating.

### 3.2.3. Heating microscopy

Heating microscopy (HM) was used to assess thermal behaviour of ACP in situ for the first time and to check whether standalone use of HM is possible for crystallization detection of ACP. HM curves are shown on Fig. 7 and their correlation with the DSC-TGA results (Fig. 8) will be discussed below.

Negligible differences in HM curves were observed for FrD samples at all synthesis end pH values, while Ov samples gave different curves. The observed cross-section area changes of sample during heat treatment partly correlated with crystallization events detected in DSC-TGA. Turns out that crystallization of ACP is accompanied by packing of particles thus decreasing cross-section area of the sample. The first cross-section area changes of the samples up to 400 °C is related to mass losses associated with loss of water as it is in TGA. Order of Ov sample HM curves as in TGA graphs (Fig. 4) – greatest cross-section area decrease in HM and mass loss in TGA is for Ov_pH 11 and the smallest for Ov_pH 8, while pH 9 and 10 have similar behaviour and lay in the middle. For FrD samples such HM graph order was not observed.

Further the next step of cross-section area decrease is related to crystallization. Here the data from both methods in the case of Ov samples were combined: 1) the T_{cryst.onset} and T_{cryst.end} from DSC were correlated with T before and after sample shrinkage in HM (Fig. 8a) and 2) the mass loss (TGA) at T_{cryst.onset} and T_{cryst.end} (DSC) and cross-section area change (HM) before and after sample shrinkage were correlated depending on synthesis end pH (Fig. 8b). In Fig. 8a close
correlation for HM temperature after sample shrinkage with DSC T_{crys.end} for samples with synthesis end pH 9–11 is observed. HM temperature before sample shrinkage with DSC T_{crys.onset} has a similar trend, however here HM underestimates the T_{crys.onset} for 100–150 °C. Fig. 8b depicts close correlation for HM cross-section area before sample shrinkage with TGA mass loss at T_{crys.onset}. Here HM cross-section area slightly overestimates the mass loss detected by TGA. Further the HM cross-section area after shrinkage of the sample with TGA mass loss at T_{crys.end} follows similar trend to previous one, but HM overestimates the TGA data by around 5%. Overall only few thermal characteristics acquirable by HM (HM T after sample shrinkage and cross-section area before sample shrinkage) are related to DSC-TGA parameters (T_{crys.end} and mass loss at T_{crys.onset}).

Data for n-HAp and R-HAp between both methods were not correlated as crystallization related phenomena were absent in DSC-TGA.

3.3. Heat treated ACP samples

Phase composition and degree of crystallinity (DOC) of heat treated ACP samples are shown on Table 2.

3.3.1. Phase and chemical analysis of heat treated samples

XRD phase analysis revealed that n-HAp and R-HAp samples consisted of HAp phase only. Drying method and pH of the synthesis have an impact on phase composition for ACP samples heated up to T_{end.cryt}, however after 1200 °C such differences were not observed. After 1200 °C the same phase composition was obtained for each pH value regardless of chosen drying method. Still, there were differences present: it was only β-TCP for pH 8–9 and HAp/β-TCP for pH 10-11. Further, the phase composition for samples at T_{end.cryt} was more diverse: only β-TCP; β-TCP and HAp; β-TCP and α-TCP; α-TCP, β-TCP and HAp. α-TCP was detected only for FrD samples precipitated at pH9, pH 10 and pH 11. All samples after both heat treatment temperatures, except FrD_pH9, contained β-TCP as the only or main crystalline phase. Main phase of FrD_pH9 was α-TCP. This demonstrates that differences in the drying process of ACP play an important role in structural development of CaPs during heat treatment, e.g., different times spent for samples in the wet state. In the case of FrD – sample was immediately frozen in liquid N2 after washing while the Ov sample stayed wet with decreasing amount of moisture until it is dry. The conclusion is that heat treatment only at high temperatures (e.g. 1200 °C) does not tell the whole story about structural differences introduced in early stages of the CaP synthesis and post-processing. This is of interest for amorphous samples in particular as in this case XRD analysis for as-synthesized materials reveals little information.

FT-IR spectra of samples after T_{crys.end} and 1200 °C are shown on Fig. S1 and Fig. S2 with absorption band identification on Tables S2 and
S3 in Supplementary data. Phosphate group absorption bands ($\nu_3$, $\nu_2$, $\nu_1$ and $\nu_3$ PO$_4^{3-}$) were identified belonging to phases identified with XRD. Interestingly, carbonate groups were detectable for samples prepared at pH 10 and pH 11 even at T$_{\text{cryst.end}}$. At 1200 °C carbonate groups were absent for all samples including n-HAp and R-HAp reference samples. Usually, loss of carbonate ions starts at 400–500 °C and is completed at 800–1200 °C [36] or between 630–1250 °C [37].

Formation of $\beta$-TCP from ACP is logical, because theoretical Ca/P molar ratio of both of them is 1.5 [38]. However, the synthesis system in this work have Ca/P of 1.67. Therefore, another phase or phases, e.g., non-stoichiometric calcium deficient HAp or biphasic mixture of HAp/ $\beta$-TCP, can form. Formation of the biphasic mixture from ACP can be explained by presence of other ions (carbonate, excess of calcium and chlorine) in synthesis medium and later in the hydrated layer [39] of ACP particle.

As carbonate leaves the structure, the Ca/(P + C) ratio increases and the HAp could form as well. Further, excess of Ca$^{2+}$ in synthesis medium facilitates CaP transformation to HAp. This was shown for brushite [33] and ACP [7]. It is known that higher Ca/P ratio in synthesis medium speeds up the transformation rate from ACP to HAp [40], therefore we assume that there will be differences in phase composition of such heat treated ACP. Further, obtaining of HAp/$\alpha$-TCP was shown by thermally decomposing CaP product precipitated from solution with Ca/P = 1.60 [41]. And when there is chlorine in synthesis system it tends to transform calcium deficient apatite into mixture of HAp and $\beta$-TCP [42].

### 3.3.2. Degree of crystallinity

DOC was calculated after T$_{\text{cryst.end}}$ and 1200 °C treatment (see Table 2). For x-ray amorphous samples DOC was assumed to be zero. Samples after 1200 °C have reached DOC of 95–100%. Samples heat treated at T$_{\text{cryst.end}}$ reached DOC of 82–100%. For FrD samples difference in DOC after T$_{\text{cryst.end}}$ and 1200 °C is approximately the same for all samples (13–16%) (see Table 2). This clearly demonstrated that end pH of the synthesis medium does not eventually affect crystallinity of developing CaP phases from ACP, and further crystallization from T$_{\text{cryst.end}}$ up to 1200 °C follows the same route. For Ov samples difference between DOC at 1200 °C run and T$_{\text{cryst.end}}$ is 4–13%. Here pH of the synthesis has slight impact on DOC through the structural differences of the as synthesized samples. At pH 8 low-crystalline CaP is obtained right after synthesis [21], therefore heat treatment of such sample up to T$_{\text{cryst.end}}$ produces samples with higher DOC that is already comparable to samples obtained at 1200 °C. The initial crystallinity in ACP speeds up the crystallization process and allows to obtain higher DOC at lower temperatures. For fully amorphous samples drying method or synthesis end pH did not affect the amount of crystalline

Fig. 7. Heating microscopy curves of oven (a) and freeze dried (b) CaP samples.

Fig. 8. (a) comparison of crystallization onset/ end temperatures from DSC and temperatures corresponding to before/after shrinkage of sample from heating microscopy (HM); (b) comparison of mass losses from TGA at onset/ end crystallization temperatures and cross-section area changes before/after sample shrinkage from HM.
fraction after heat treatment. Highly crystalline CaPs from ACP can be obtained roughly right after T<br>Cryst.end and further heat treatment up to 1000 °C or more is unnecessary if for example better mechanical properties are not of interest as well.

4. Conclusions

Study on crystallization of carbonated amorphous calcium phosphates obtained from solutions in pH range of 8–11 and air dried at 80 °C or freeze dried, increase the overall knowledge on crystallization of calcium phosphates. Synthesis pH affects the structure of as synthesized ACP and leads to differences in crystallization. Regardless of pH and drying method, all studied ACP transformed into crystalline phases upon heating, with onset of the process over 600–650 °C. For the first time it was shown that, heating of ACP at crystallization end temperature without temperature hold produces material with DOC = 82–90%; higher temperatures and/or hold times are needed to obtain fully crystalline calcium phosphate materials, thus reconsidering time and cost of production.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jeurceramsoc.2018.11.003.

References


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