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# Exploring the effect of $\text{Mg}^{2+}$ substitution on amorphous calcium phosphate nanoparticles

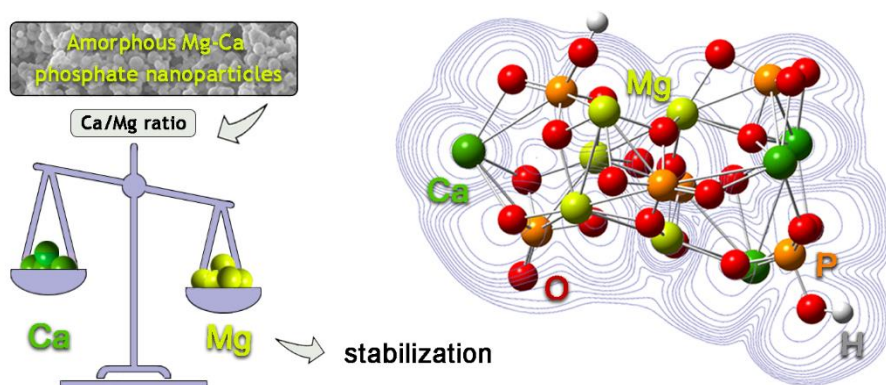
Rita Gelli<sup>a,b</sup>, Lorenzo Briccolani-Bandini<sup>a</sup>, Marco Pagliai<sup>a</sup>, Gianni Cardini<sup>a</sup>, Francesca Ridi<sup>a,b\*</sup>,  
Piero Baglioni<sup>a,b</sup>

a) Department of Chemistry “Ugo Schiff”, University of Florence, via della Lastruccia 3,  
50019 Sesto Fiorentino, Florence, Italy

b) CSGI, Center for Colloid and Surface Science, via della Lastruccia 3, 50019 Sesto  
Fiorentino, Florence, Italy

\*Corresponding author: Francesca Ridi, E-Mail francesca.ridi@unifi.it

## Graphical abstract



**Abbreviations:** ACP: Amorphous Calcium Phosphate; AMCP: Amorphous Magnesium Calcium Phosphate; CaP: Calcium Phosphate; HA: Hydroxyapatite; XRD: X-Ray Diffraction; PDF: Powder Diffraction Files; ICDD: International Centre for Diffraction Data; FWHM: Full Width at Half Maximum; ATR-FTIR: Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy; FE-SEM: Field Emission-Scanning Electron Microscopy; ICP-OES: Inductively Coupled Plasma-Atomic Emission Spectrometry; SDT: Simultaneous Thermogravimetry/Differential Scanning Calorimetry; TCP: Tri-Calcium Phosphate; TMCP: Mg-substituted Tri-Calcium Phosphate.

## **Abstract**

### *Hypothesis*

The study of Amorphous Calcium Phosphate (ACP) has become a hot topic due to its relevance in living organisms and as a material for biomedical applications. The preparation and characterization of Mg-substituted ACP nanoparticles (AMCP) with tunable Ca/Mg ratio is reported in the present study to address the effect of  $\text{Mg}^{2+}$  on their structure and stability.

### *Experiments*

AMCPs particles were synthesized by precipitation of the precursors from aqueous solutions. The particles were analyzed in terms of morphology, crystallinity, and thermal stability, to get a complete overview of their physico-chemical characteristics. Computational methods were also employed to simulate the structure of ACP clusters at different levels of  $\text{Mg}^{2+}$  substitution.

### *Findings*

Our results demonstrate that AMCP particles with tunable composition and crystallinity can be obtained. The analysis of the heat-induced crystallization of AMCP shows that particles' stability depends on the degree of  $\text{Mg}^{2+}$  substitution in the cluster, as confirmed by computational analyses. The presented results shed light on the effect of  $\text{Mg}^{2+}$  on ACP features at different structural levels and may be useful guidelines for the preparation and design of AMCP particles with a specific Ca/Mg ratio.

## **Keywords**

Amorphous calcium phosphates; Magnesium; Posner's cluster; Nanoparticles; Nanostructure; Morphology; Stability; Thermal Analysis; Simulations.

## **1 Introduction**

Amorphous Calcium Phosphate (ACP) is particularly relevant among the different forms of Calcium Phosphate (CaP) present in nature and in living organisms [1]. Given its chemical and structural

versatility, ACP has a dramatic biological importance as a transient intermediate in the formation of the apatitic inorganic matrix of bones and teeth and in the formation of other types of functional structures such as casein micelles, calciprotein particles and amorphous nanoparticles in the small intestine [2]. Despite its amorphous nature, ACP displays a short-range order corresponding to the so-called “Posner’s Cluster”  $\text{Ca}_9(\text{PO}_4)_6$  [3], which is thermodynamically unstable and spontaneously converts into a crystalline form of CaP, such as hydroxyapatite (HA,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ), brushite ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ ) or octacalcium phosphate ( $\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$ ) [4]. The intrinsically metastable and dynamic nature of ACP and, in general, of amorphous materials, makes them very versatile and attractive for several applications in the biomedical field, including drug delivery [5–7], dentistry [8,9] and bone regeneration [10,11].

Notwithstanding the importance of pure ACP, the role that  $\text{Mg}^{2+}$  exerts on ACP features and stability is receiving considerable attention from the scientific community, due to the key functions exerted in biological organisms. On the average, an adult human body contains about 24 g of  $\text{Mg}^{2+}$ , about half stored in bone tissue [12]. Magnesium levels in our organism are tightly controlled, in order to prevent the multiple pathologic situations caused by its deficiency, such as low bone mass, reduced bone growth, osteoporosis and increased skeletal fragility [13–15]. Besides  $\text{Mg}^{2+}$  role in cellular metabolism [16] and bone formation [17], it has recently emerged that *in vivo* its interaction with ACP results in the formation of AMCP (Amorphous Magnesium Calcium Phosphate) particles both in the small intestine [18] and in enamel [19,20].

Long before the full recognition of the biological importance of  $\text{Mg}^{2+}$  interplay with ACP, its effect on the formation and crystallization of CaP was thoroughly investigated starting from the sixties [21–29]. In a seminal study from Boskey and Posner, the conversion kinetic of ACP to HA in the presence of  $\text{Mg}^{2+}$  was investigated, revealing that an increase in Mg/Ca ratio from 0 to 0.04 extends the amorphous phase’s lifetime, from 4 to 9 times depending on the temperature [21]. The stabilizing effect of  $\text{Mg}^{2+}$  was ascribed to a decrease in the solubility of ACP, while other authors suggest that the formation of Mg-phosphate complexes is responsible for the enhancement of the amorphous’

lifetime [22]. As an alternative mechanism for the stabilization, Abbona and Baronnet hypothesize that the initial  $\text{Mg}^{2+}$  substitution for  $\text{Ca}^{2+}$  in Posner's clusters induces mechanical strains and hinders HA nucleation, in addition to the effect due to  $\text{Mg}^{2+}$  adsorption onto the surfaces of ACP and of apatite crystallites [26]. Despite the large number of studies carried out from the sixties, the role that  $\text{Mg}^{2+}$  exerts on ACP is still an open issue [30–37]. Yang *et al.* suggested that the formation of Mg-phosphate ion pairs reduces the thermodynamic driving force for ACP nucleation and the subsequent crystallization to HA [30], while others proposed that  $\text{Mg}^{2+}$  might also have an effect in increasing HA's solubility [33]. A study by Ding *et al.* addressed the effect of  $\text{Mg}^{2+}$  ions on the different stages of HA precipitation from ACP, which can either be adsorbed or incorporated into the ACP precursor particles [31]. Other investigations took advantage of combined experimental-computational approaches to tackle this issue [32]. However, the studies conducted so far are not able to shed light on the role of  $\text{Mg}^{2+}$  in the stabilization of the amorphous phase, likely resulting from multiple simultaneous mechanisms with implications in the *in vivo* biomineralization processes.

It is important to point out that  $\text{Mg}^{2+}$  role is meaningful not only for the crystallization pathway of ACP, but also for the structural features of the amorphous phase itself, as  $\text{Mg}^{2+}$  can be incorporated in the amorphous cluster to form AMCP particles. Few reports describe the preparation of AMCPs with different Ca/Mg ratio [24,38], sometimes coupled with the study of their evolution in simulated body fluids [39,40]. However, little attention is devoted to the characterization of the features of the different amorphous phases, and to the understanding of the  $\text{Ca}^{2+}$  ions substitution in the amorphous cluster. The control of AMCP particles with tailored Ca/Mg ratio and the knowledge of their structural features is of fundamental importance to prepare particles with predetermined stability, to be used in several applications as the treatment of dentin hypersensitivity [41,42], as well as to reproduce the endogenous nanoparticles present in the small intestine [43].

The aim of this study is the investigation of the variation of the Ca/Mg ratio and the total cations concentration on the properties of the precipitated amorphous phases. A systematic analysis was conducted by performing 15 different syntheses in which the concentrations of the reacting solutions

were varied to test 5 different Ca/Mg ratios (0.4, 0.7, 1, 2 and 3), each at 3 different  $\text{Mg}^{2+}$  concentrations (10, 50 and 100 mM). The obtained particles were analyzed in terms of crystallinity, morphology, elemental composition, and thermal stability, while computational approaches were used to gain insights into the structure of these particles at the atomic level, disclosing new insights into the role of  $\text{Mg}^{2+}$  substitution in ACP.

## **2 Materials and methods**

### **2.1 Materials**

Calcium chloride ( $\text{CaCl}_2$ ,  $\geq 93\%$ ), Magnesium chloride hexahydrate ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\geq 99\%$ ), Sodium chloride ( $\text{NaCl}$ ,  $\geq 99\%$ ) and NaOH pellets ( $\geq 99\%$ ) were purchased from Sigma-Aldrich (Milan, Italy). Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ,  $\geq 99\%$ ), and sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ,  $\geq 99\%$ ) were obtained from Carlo Erba Reagents (Milan, Italy). Milli-Q grade water (Millipore, resistivity  $18.2 \text{ M}\Omega \cdot \text{cm}$  at  $25^\circ\text{C}$ ) was used during all the experiments.

### **2.2 Preparation of AMCP**

AMCPs were prepared by precipitation from aqueous solution, according to a method readapted from the literature [24]. 10 mL of solution A (from A1 to A15, see Table 1) were mixed with 10 ml of solution B (150 mM  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  + 50 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ), at  $37^\circ\text{C}$ . Immediately after mixing, the pH was adjusted at 7.40 by means of 1 M NaOH. It was observed that the higher the concentration of the reactants used, the higher the turbidity of the reacting medium. After 15 min of reaction, the particles were collected by means of filtration, using a Millipore vacuum filtration system equipped with mixed cellulose esters filters (Millipore, pore size  $0.45 \mu\text{m}$ ). Immediately after filtration, the filters were placed in plastic test tubes and frozen in liquid nitrogen. The powders were then freeze-dried for 24 h, at  $-55^\circ\text{C}$  and  $\sim 30 \text{ mTorr}$  (VirTis BenchTop freeze-dryer, NY, USA) and stored in the freezer ( $-18^\circ\text{C}$ ), tightly closed to prevent any influence from environmental humidity [11].

**Table 1.** Composition of the solutions used for the syntheses.

| Sample | CaCl <sub>2</sub><br>[mM] | MgCl <sub>2</sub><br>[mM] | NaCl<br>[mM] | Ca/P  | Mg/P | Ca/Mg |
|--------|---------------------------|---------------------------|--------------|-------|------|-------|
| A1     | 4                         | 10                        | 135          | 0.02  | 0.05 | 0.4   |
| A2     | 20                        | 50                        | 135          | 0.1   | 0.25 |       |
| A3     | 40                        | 100                       | 135          | 0.2   | 0.5  |       |
| A4     | 7                         | 10                        | 135          | 0.035 | 0.05 | 0.7   |
| A5     | 35                        | 50                        | 135          | 0.175 | 0.25 |       |
| A6     | 70                        | 100                       | 135          | 0.35  | 0.5  |       |
| A7     | 10                        | 10                        | 135          | 0.05  | 0.05 | 1     |
| A8     | 50                        | 50                        | 135          | 0.25  | 0.25 |       |
| A9     | 100                       | 100                       | 135          | 0.5   | 0.5  |       |
| A10    | 20                        | 10                        | 135          | 0.1   | 0.05 | 2     |
| A11    | 100                       | 50                        | 135          | 0.5   | 0.25 |       |
| A12    | 200                       | 100                       | 135          | 1     | 0.5  |       |
| A13    | 30                        | 10                        | 135          | 0.15  | 0.05 | 3     |
| A14    | 150                       | 50                        | 135          | 0.75  | 0.25 |       |
| A15    | 300                       | 100                       | 135          | 1.5   | 0.5  |       |

## 2.3 Characterization techniques

### 2.3.1 X-Ray Diffraction (XRD)

XRD patterns were collected using a D8 Advance with DAVINCI design (Bruker, Milan, Italy), using as X-ray source the Cu K $\alpha$  radiation (wavelength  $\lambda=1.54$  Å), at 40 kV and 40 mA, a  $2\theta$  range of 10°-50°, a step size of 0.03°, and a time/step of 0.3 s. Samples were grinded with agate mortar and pestle

and flattened onto a Si zero-background sample holder. Peaks were assigned using the Powder Diffraction Files (PDF) of the ICDD database (International Centre for Diffraction Data).

Scherrer equation (Eq. 1) was used to calculate the crystalline domain size (D, nm):

$$D = \frac{\kappa\lambda}{\beta\cos(\theta)} \quad \text{Eq. 1}$$

where  $\kappa$  is the Scherrer constant, typically considered 0.9,  $\lambda$  is the X-Ray wavelength (nm),  $\beta$  is the FWHM (Full Width at Half Maximum) in radians and  $\theta$  is the diffraction angle in radians [44]. The FWHM were obtained from the Multi-Peak Fitting routine of the software IgorPro, considering a logcubic function as baseline.

### **2.3.2 Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR)**

ATR-FTIR spectra on the freeze-dried samples were acquired by means of a Nexus Thermo-Nicolet 870 FT-IR spectrophotometer (Madison, WI, USA) equipped with a MCT detector and a Golden Gate. The spectra were collected in the 4000-650  $\text{cm}^{-1}$  range, with 128 scans and resolution 2  $\text{cm}^{-1}$ .

### **2.3.3 Field Emission-Scanning Electron Microscopy (FE-SEM)**

FE-SEM micrographs were collected by means of a Zeiss SIGMA FE-SEM (Carl Zeiss Microscopy GmbH, Jena, Germany). The powders were placed over aluminum stubs by means of conductive tape. The use of a Field Emission source, together with a low accelerating voltage (2 kV), a reduced sample-detector distance (~2 mm) and the In-Lens detector allowed for the imaging of non-conductive samples at high magnification. The size distribution curves were obtained with the software ImageJ by measuring about 200 particles *per* sample.

### **2.3.4 Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-OES)**

The amount of phosphorous in the samples was determined in triplicate by means of an Agilent 720-ES Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-OES) equipped with a pneumatic nebulizer and a cyclonic spray chamber. Before the analyses, about 1.0 mg of each sample was dissolved in 10  $\mu\text{L}$  of HCl, brought to 10 mL with ultrapure water and added with 1.0 ppm of Ge, used as an internal standard to correct any matrix effect. The calibration lines were obtained by

preparing the standard solutions starting from commercial standards certified at 1000 ppm. Ge line used for the correction was at 209.426 nm. After each measurement, the system was washed with a 2% HNO<sub>3</sub> solution.

### **2.3.5 Ionic chromatography**

The amount of calcium, magnesium and sodium was determined by means of ionic chromatography using a Thermo Dionex ICS-1000 ion chromatograph with a CG-12A guard column and a CS-12A analytical column. The same solutions prepared for ICP-OES measurements were analyzed. An electrochemical suppression of the background conductivity with a current of 160 mA was used, together with an injection loop of 25 µL with a conductivity detector. The element used for the separation was H<sub>2</sub>SO<sub>4</sub> 23 mM. The calibration lines of the individual ions were constructed for subsequent dilutions starting from standard certified solutions at 1000 ppm.

For both ICP-OES and ionic chromatography, to evaluate the reproducibility of the results, on selected samples the measurements were conducted on 3 specimens prepared by repeating the same synthesis three times. In all cases, the standard deviation of the three measurements lead to atomic ratios uncertainties < 0.1.

### **2.3.6 Simultaneous Thermogravimetry/Differential Scanning Calorimetry (SDT)**

Thermal analysis was conducted with a SDT Q600 from TA Instruments (New Castle, DE, USA), which allows for the simultaneous monitoring of the weight loss signal (TGA curve) and the heat flow profile (DSC curve). Samples were placed in an alumina pan and measured in N<sub>2</sub> atmosphere (flow rate 100 mL/min) from room temperature to 1000 °C, at 10 °C/min. The crystallization T is expressed as average ± standard deviation of 3 measurements conducted on 3 replicas of each synthesis.

### **2.3.7 Computational Details**

To investigate the structural properties of selected magnesium and calcium phosphate clusters, DFT calculations have been performed at the B3LYP/6-31G(d) level of theory with the Gaussian suite of programs[45] using tight criteria. The initial molecular structure has been obtained by optimizing the

atomic positions of a brushite crystal (Figure S1) with the CP2K suite of programs [46]. The crystal structure calculations have been performed with the Quick Step module employing the LDA exchange and correlation functional within the Padé approximation [47]. The wave functions for each atomic species have been described through a DZVP-MOLOPT basis set [48], whereas the electron density has been represented with a plane wave expansion with a 340 Ry cutoff. The supercell is made up by  $3 \times 1 \times 3$  unit cells. The calculations show a complete agreement between the computed and experimental structure [49]. Moreover, two phosphate ions have been hydrogenated according to the experimental results (see the characterization of the obtained particles in the Results section) and some calcium ions have been progressively replaced by magnesium ions, as summarized in Table 2, where the chemical compositions of the studied clusters are collected. In the Table, the simulated clusters are identified as An', where the presence of the apex indicates a chemical composition of the cluster close to that obtained in the synthesized samples An (Table 3 in the Results and Discussion section). The "N\_Mg" or "N\_Ca" label indicates the number of Ca or Mg ions present in the simulated cluster, respectively.

**Table 2.** Different Ca/Mg, Ca/P and Mg/P ratios for the analyzed Posner's clusters. Four different arrangements have been chosen for each sample

| Simulated cluster <sup>†</sup> | Ca/Mg | Ca/P | Mg/P | N_Ca <sup>††</sup> | N_Mg <sup>†††</sup> |
|--------------------------------|-------|------|------|--------------------|---------------------|
| Ca <sub>0</sub>                | 0     | 0    | 1.5  | 0                  | 9                   |
| A0'                            | 0.12  | 0.16 | 1.33 | 1                  | 8                   |
| A3'                            | 0.5   | 0.5  | 1    | 3                  | 6                   |
| A6'                            | 0.8   | 0.66 | 0.83 | 4                  | 5                   |
| A1'                            | 2     | 1    | 0.5  | 6                  | 3                   |
| A7'                            | 3.5   | 1.16 | 0.33 | 7                  | 2                   |
| Mg <sub>0</sub>                | --    | 1.5  | 0    | 9                  | 0                   |

† Simulated clusters are identified with the An' label, which indicates an elemental composition close to the synthesized An samples. The simulated clusters Ca<sub>0</sub> and Mg<sub>0</sub> refer to clusters containing only Ca or Mg atoms, respectively.

†† Number of Ca atoms in the simulated cluster.

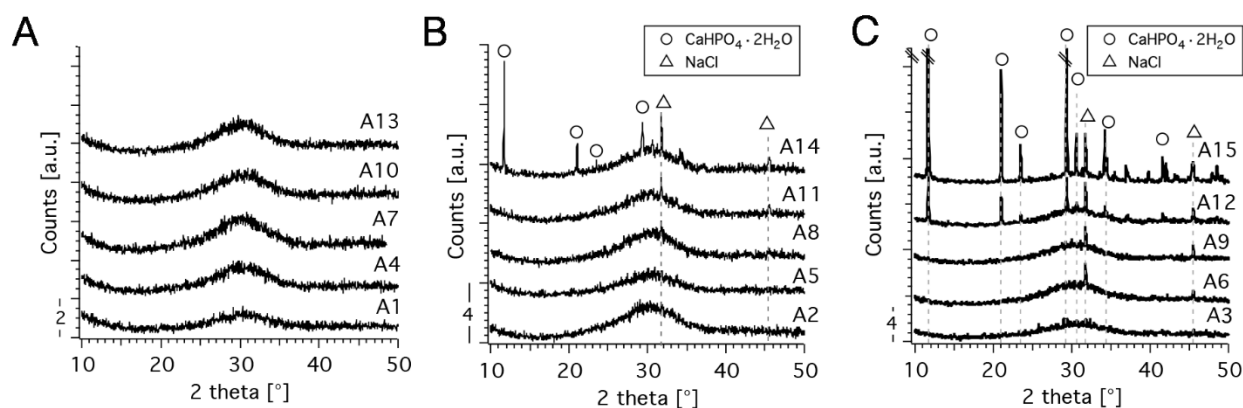
††† Number of Mg atoms in the simulated cluster.

The optimized structures have been subsequently analyzed to obtain information on the distances between the oxygen atoms and metal ions. The interaction energy between one selected Mg<sup>2+</sup> ion and the remaining cluster has been calculated with the counterpoise correction method [50,51].

### 3 Results and discussion

#### 3.1 Crystallinity

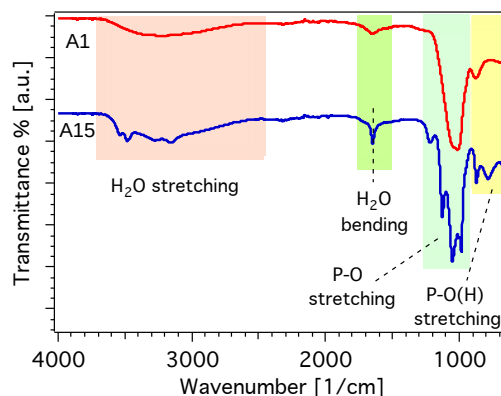
The synthesized particles were analyzed by means of X-Ray Diffraction, in order to assess their amorphous nature. The diffractograms, reported in Figure 1, reveal that in almost all cases the formation of completely amorphous particles is achieved, as confirmed by the hump at ~30° that is the fingerprint of amorphous calcium phosphates [52,53]. On the other hand, samples A12, A14 and A15 also display sharp diffraction peaks which reveal the presence of brushite (CaHPO<sub>4</sub>·2H<sub>2</sub>O, dicalcium phosphate dihydrate); this is particularly evident for the A15 sample, prepared with the highest Ca<sup>2+</sup> concentration. The average crystallite size for brushite crystals, calculated using the Scherrer equation (Eq. 1), is (61 ± 11) nm for A12, (60 ± 16) nm for A14 and (65 ± 15) nm for A15: these results suggest that no modifications in the crystallite size of brushite occur due to the different synthetic conditions. It is worth noting that despite the 7.40 pH used for the synthesis, the relatively acidic phase brushite is formed, due to the presence of Mg<sup>2+</sup> ions in solution, which strongly hinder the formation of hydroxyapatite [4]. The small peaks at 31.7° and 45.5° in samples A11, A14, A6, A9, A12 and A15 are compatible with residual NaCl from the synthetic medium (average crystallite size of NaCl: 41 ± 15 nm).



**Figure 1.** XRD patterns of the synthesized particles. A)  $[\text{Mg}^{2+}] = 10$  mM series, B)  $[\text{Mg}^{2+}] = 50$  mM series, C)  $[\text{Mg}^{2+}] = 100$  mM series. All diffractograms correspond to AMCP, except for A12, A14 and A15 which also contain  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (brushite, PDF: 09-0077, circle markers). The triangular markers correspond to NaCl crystals (halite, PDF: 01-0993). Diffractograms are offset for display purposes.

The findings from XRD analysis were corroborated by ATR-FTIR analysis: the spectra for all samples are reported in Figure S2 in the Supplementary Material. The spectra for A1, representative of amorphous samples, and A15, representative of brushite, are shown in Figure 2. All spectra, except A15, display vibrational peaks characteristic of amorphous phosphates, namely: *i.* broad band at  $3400\text{--}2600\text{ cm}^{-1}$  due to water's O-H stretching; *ii.* peak at  $1653\text{ cm}^{-1}$  due to water bending; *iii.* intense peak at about  $1000\text{ cm}^{-1}$  due to phosphate stretching and *iv.* peak at  $877\text{ cm}^{-1}$  due to H–O(P) stretching in  $\text{HPO}_4^{2-}$  ions [11,23,52]. The A15 spectrum presents the characteristic sharp and defined peaks typical of brushite [54,55].

XRD and FTIR analyses show that the final product can be pure amorphous particles or a mix of amorphous particles and brushite crystals, depending on the initial concentration and  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ratio used in the synthesis.

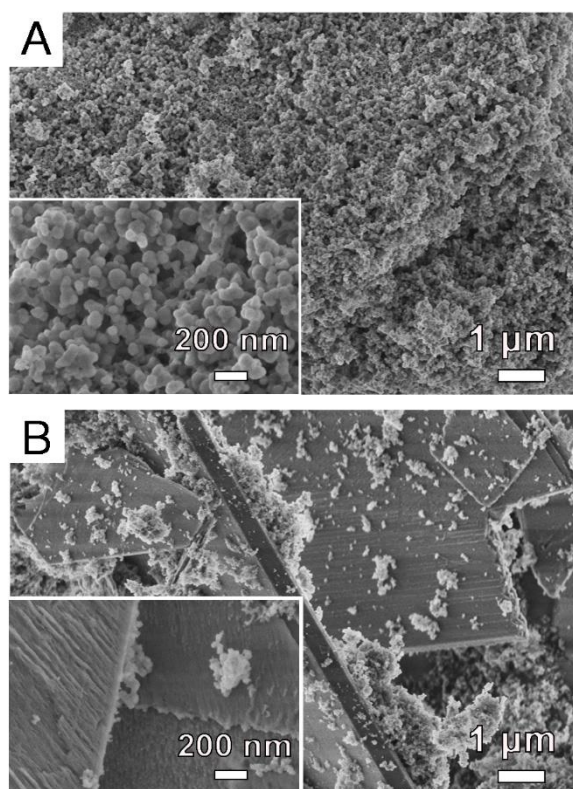


**Figure 2.** ATR-FTIR spectra of A1 (top, red curve) and A15 (bottom, blue curve).

### 3.2 Morphology

The morphology of the synthesized particles was investigated by means of scanning electron microscopy: the micrographs of A1 and A15 are reported as an example in Figure 3A and B, respectively, whereas a complete overview of all the samples can be found in Figure S3 and S4. The nano-sized amorphous particles present spherically shaped particle units that aggregate. The size distribution curves, see the insets in Figure S3, show that the single unit constituting the aggregate is polydisperse with an average diameter from about 40 nm to 80 nm. Despite the large polydispersity, the data reported in Figure S3 show that the dimension of the single units decreases when the total concentration of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  is increased with respect to phosphate at a given Ca/Mg ratio, in agreement to the nucleation and growth theory [56].

In addition to amorphous aggregates, samples A12, A14 and A15 display well-defined platelets typical of well-crystalline brushite (see Figure S4 and Figure 3B), in agreement with XRD findings.



**Figure 3.** A) FE-SEM micrograph of sample A1, showing AMCP particles; B) FE-SEM micrograph of sample A15, which shows the presence of AMCP together with brushite platelets. In the insets, high magnification micrographs are shown.

### 3.3 Elemental composition

The elemental composition of the particles was investigated by means of ICP-OES (for the quantification of P) and ionic chromatography (for the quantification of Ca, Mg and Na). The atomic ratios are reported in Table 3 and Figure S5, while the Ca/Mg ratio as a function of the Ca/Mg ratio used in the synthesis of the particles is shown in Figure 4.

**Table 3.** Elemental ratios of the particles as obtained by means of ICP-OES and ionic chromatography. The error associated to each ratio is  $< 0.1$  (see section 2.3.5).

|    | Ca/Mg | Ca/P | Mg/P | (Ca+Mg)/P | Na/P |
|----|-------|------|------|-----------|------|
| A1 | 2.13  | 0.82 | 0.38 | 1.20      | 0.24 |
| A2 | 0.97  | 0.60 | 0.62 | 1.23      | 0.29 |
| A3 | 0.63  | 0.45 | 0.71 | 1.17      | 0.29 |

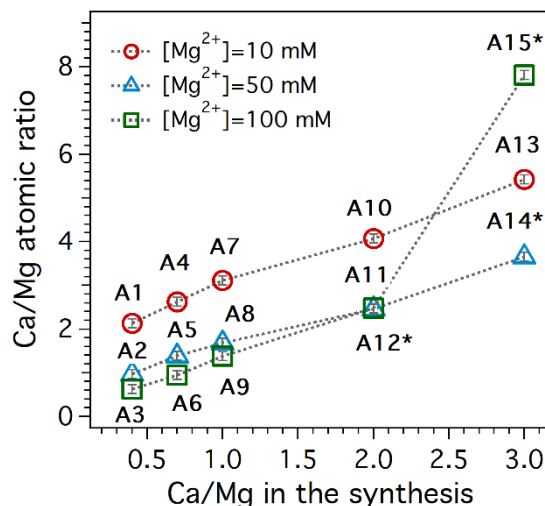
|      |      |      |      |      |      |
|------|------|------|------|------|------|
| A4   | 2.62 | 0.87 | 0.33 | 1.21 | 0.23 |
| A5   | 1.39 | 0.65 | 0.47 | 1.12 | 0.27 |
| A6   | 0.95 | 0.58 | 0.62 | 1.20 | 0.30 |
| A7   | 3.11 | 0.92 | 0.30 | 1.21 | 0.25 |
| A8   | 1.68 | 0.75 | 0.44 | 1.19 | 0.29 |
| A9   | 1.38 | 0.74 | 0.54 | 1.27 | 0.30 |
| A10  | 4.07 | 0.97 | 0.24 | 1.21 | 0.23 |
| A11  | 2.46 | 0.87 | 0.35 | 1.22 | 0.27 |
| A12* | 2.48 | 0.96 | 0.39 | 1.35 | 0.26 |
| A13  | 5.42 | 1.02 | 0.19 | 1.21 | 0.26 |
| A14* | 3.65 | 0.93 | 0.26 | 1.19 | 0.25 |
| A15* | 7.81 | 1.16 | 0.15 | 1.31 | 0.20 |

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\*samples also containing brushite.

The Ca/Mg in the precipitated particles is always higher than that of the solutions used to prepare them, suggesting the preferential incorporation of  $\text{Ca}^{2+}$  with respect to  $\text{Mg}^{2+}$ , which is related to the lower solubility of CaPs with respect to MgPs [57]. In any case, we observe that an increase in the Ca/Mg ratio of the synthetic solutions always corresponds to an increase in the Ca/Mg ratio of the precipitated particles. Moreover, for a given Ca/Mg ratio the amount of  $\text{Ca}^{2+}$  incorporated in the particles is higher in the more diluted regimes. The Ca/Mg ratio of samples containing brushite is not a reliable guess of the composition of the amorphous particles, as the presence of brushite crystalline phase (containing only  $\text{Ca}^{2+}$ ) induces an overestimate of the Ca/Mg ratio (samples A12, A14 and A15, marked with a \* in Figure 4) especially for A15, which is the most crystalline sample (see Figure 1C). It is interesting that above a certain Ca/Mg threshold ( $[\text{Mg}^{2+}]=50$  mM) an increase in the total cations amount does not reflect on a different Ca/Mg ratio in the particles. These results suggest that, despite almost all particles display an amorphous nature, a different amount of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  is incorporated within their structure, disclosing a simple guideline to prepare amorphous particles with a tunable composition. A small and almost constant amount of Na is also present in the particles, likely due to residual traces of NaCl from the synthetic medium in the samples (as already observed

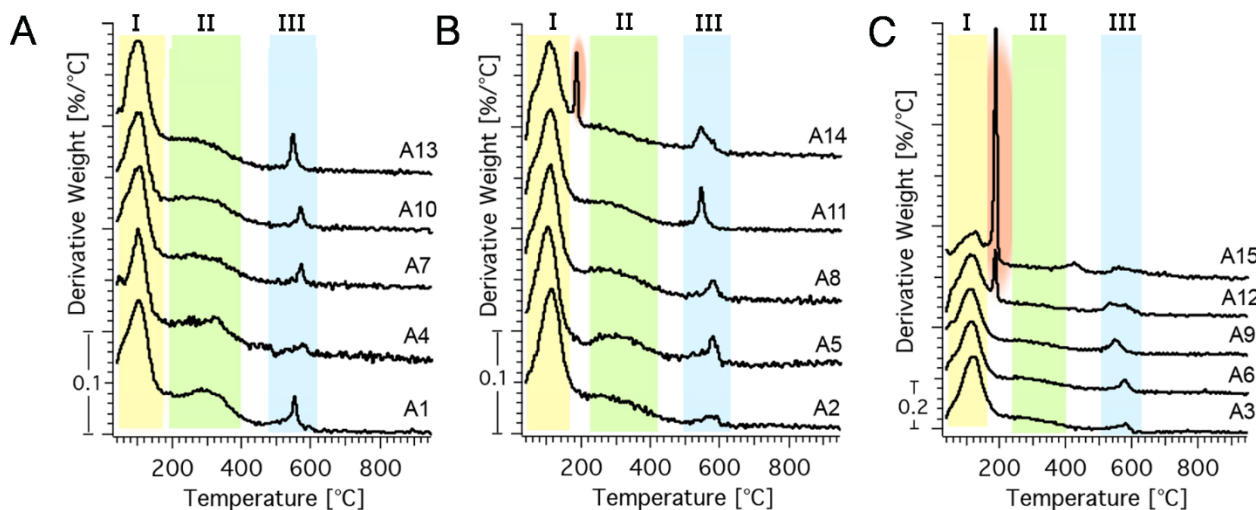
in the XRD patterns of some samples in Figure 1). Finally, the (Ca+Mg)/P ratio always smaller than 1.5 suggests that some hydrogenphosphate ions are present in the lattice, consistently with FTIR findings (see Figure 2).



**Figure 4.** Plot of the Ca/Mg atomic ratio obtained with ICP-OES/ionic chromatography vs the Ca/Mg molar ratio used in the synthesis. Samples marked with \* also contain brushite, thus their Ca/Mg ratio may include not only the contribution of AMCP.

### 3.4 Thermal behavior

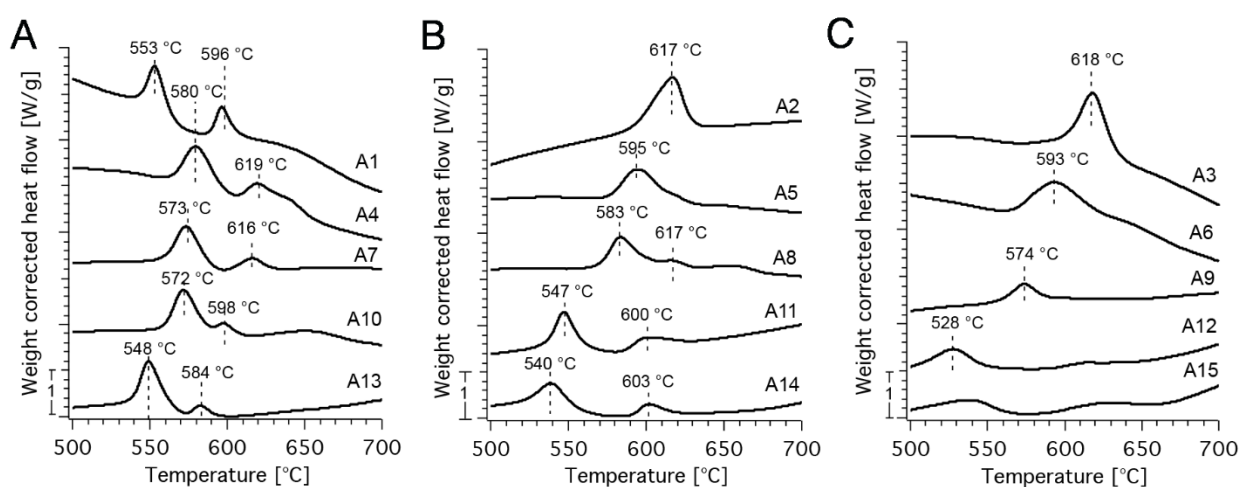
Some peculiar structural information can be obtained by analyzing the thermal degradation of the particles and the simultaneous heat flow profile by means of SDT. The derivative thermograms, reported in Figure 5, show that the degradation of the amorphous particles occurs in three different stages: *i.* the loss of loosely bound water (yellow region in the figure, step I), *ii.* the loss of strongly bound internal water (green region, step II) and *iii.* weight loss occurring in correspondence of the crystallization process (blue region; step III) [58,59]. Samples A12, A14 and A15 also display a very sharp weight loss centered at 187 °C, due to the loss of brushite's two molecules of crystallization water ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \rightarrow \text{CaHPO}_4 + 2\text{H}_2\text{O}$ , orange area in Figure 5) [60].



**Figure 5.** Derivative thermogravimetric curves of the samples under investigation; A)  $[\text{Mg}^{2+}] = 10 \text{ mM}$  series, B)  $[\text{Mg}^{2+}] = 50 \text{ mM}$  series, C)  $[\text{Mg}^{2+}] = 100 \text{ mM}$  series. The yellow (I), green (II) and blue (III) regions respectively indicate the weight losses of: I) loosely bound water, II) strongly bound water and III) weight loss occurring during crystallization. The orange circles indicate the weight loss diagnostic of brushite. All the thermograms are offset for display purposes.

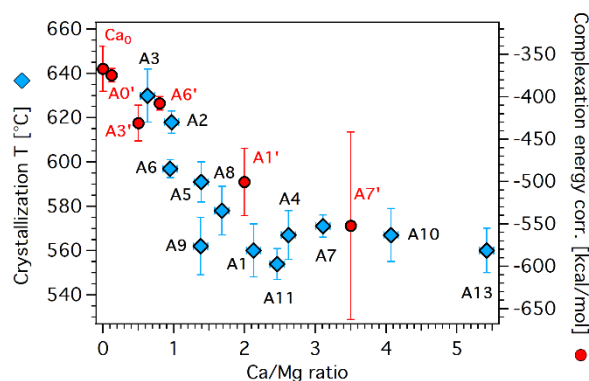
The heat flow profiles reported in Figure 6 show that amorphous particles undergo a crystallization process at a  $T$  ranging from  $540 \text{ }^{\circ}\text{C}$  to  $630 \text{ }^{\circ}\text{C}$ . The observation of crystallization events in amorphous materials is an interesting tool to gain insights into their features [61–64]. Here, the fact that samples display a different number and location of peaks suggests that structural differences among amorphous calcium-magnesium phosphate samples are present. Samples A2 and A3 display one sharp peak above  $600 \text{ }^{\circ}\text{C}$ , whereas A5, A9 and A6 samples display a single but broadened peak at lower temperatures; all other samples crystallize into two distinct steps. Samples A12, A14 and A15, containing brushite, were not considered for the analysis of the amorphous crystallization. Samples A2, A7, A8 and A10 were analyzed as case studies to understand the crystalline phase at a temperature above the crystallization peaks. The XRD patterns, reported in Figure S6, show that sample A2 converts to crystalline sodium calcium magnesium phosphate with a whitlockite-like structure ( $\text{Ca}_9\text{MgNa}(\text{PO}_4)_7$ , PDF n° 88-0797) and small amounts of  $\text{Ca}_9\text{Mg}_7\text{Na}_2(\text{PO}_4)_6$  (PDF n° 47-1763). The pattern of samples A7, A8 and A10 recovered after the first crystallization peak matches the pattern of  $\text{Ca}_9\text{MgNa}(\text{PO}_4)_7$ , whereas the small peaks which appear after the second peak are compatible with

the conversion of residual amorphous material to pyrophosphates ( $\text{Mg}_2\text{P}_2\text{O}_7$ , PDF 01-0866 and  $\text{Na}_2\text{MgP}_2\text{O}_7$ , PDF 48-0574). According to literature reports, pure amorphous calcium phosphate converts to  $\alpha$ -TCP or  $\beta$ -TCP above 600 °C, depending on the preparation conditions [58,61,65–67]. When  $\text{Mg}^{2+}$  ions are present in the lattice, the formation of  $\beta$ -TMCP (Mg-substituted TCP), which has a diffraction pattern similar to the detected phase, was observed [38]. The substitution of divalent cations on ACP is analyzed in a recent work [68], in which it is shown that the  $\text{Mg}^{2+}$  content in ACP affects the thermal behavior, promoting the formation of  $\beta$ -TCP over  $\alpha$ -TCP at lower temperatures.



**Figure 6.** Weight corrected heat flow profiles (exo up) of A)  $[\text{Mg}^{2+}] = 10 \text{ mM}$  series, B)  $[\text{Mg}^{2+}] = 50 \text{ mM}$  series, C)  $[\text{Mg}^{2+}] = 100 \text{ mM}$  series. The curves are offset for display purposes.

This work shows that the different crystallization temperatures observed in the AMCP samples do not correspond to the conversion towards different phases. A very interesting outcome arises from the observation of the plot of the crystallization temperature (maximum of the first peak) vs the particles' Ca/Mg ratio, reported in Figure 7 (left y axis), which reveals that the lower the Ca/Mg ratio of the particles, the higher the crystallization temperature. This assumption holds up to a Ca/Mg ratio of about 2. Therefore  $\text{Mg}^{2+}$ , in addition to its known stabilizing action in the conversion of amorphous to crystalline calcium/magnesium phosphates in solution [21,36], promotes the stabilization of the amorphous phase against heating when incorporated in the lattice of the particles.



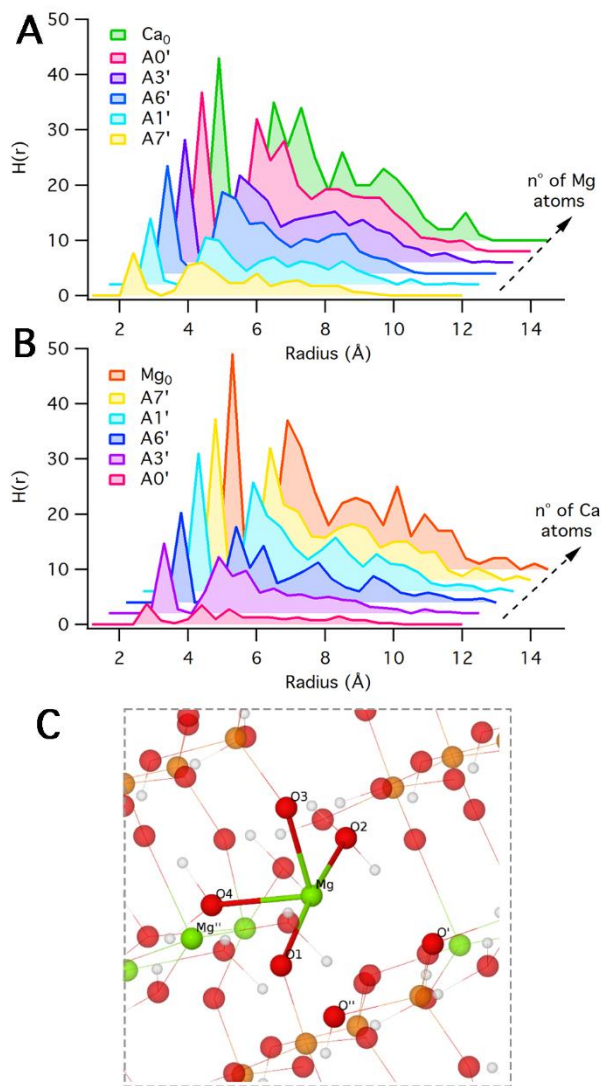
**Figure 7.** Plot of the crystallization  $T$  (1<sup>st</sup> peak maximum) vs the Ca/Mg ratio (left y axis). The blue diamond markers represent the average values  $\pm$  standard deviations (see the materials and methods section). The plot also shows the counterpoise correction energy (red dots) as a function of Ca/Mg ratio. The error bars in calculations progressively enlarge due to the limit in configuration sampling.

### 3.5 DFT calculations

These conclusions are further corroborated by the computational analyses. The interaction energies between the inner site of  $\text{Mg}^{2+}$  ion and the remaining Posner's cluster for different Ca/Mg ratio were calculated and are reported in Figure 7 (right y axis). For the homogeneous system  $\text{Ca}_0$  (only magnesium cations) the interaction energy of the inner site with the cluster is approximately of -357 kcal/mol. Instead, in the case of the cluster with two  $\text{Mg}^{2+}$  and seven  $\text{Ca}^{2+}$  ( $\text{A7}'$ , see Table 2), the counterpoise correction energy is -552 kcal/mol. The obtained trend confirms the stabilizing contribute of the  $\text{Mg}^{2+}$  cation with the increasing of calcium concentration. In fact, the binding energy in Posner's cluster increases (in absolute value) with calcium content. Qualitatively, this trend can be rationalized considering the greater coordination effect due to the higher hardness for magnesium than calcium. This determines a reduction of intermolecular distances, also considering those involving  $\text{Mg}^{2+}$  ions. In this respect, the electrostatic repulsion is greater in cluster with higher magnesium content and consequently the binding energy is lower.

These conclusions are supported by the analysis of the Mg-O distances of the clusters where the  $\text{Ca}^{2+}$  ions are replaced by  $\text{Mg}^{2+}$  ions (see computational details in 2.3.7). An increase of the magnesium content in the cluster determines a progressive stabilization of the interactions at Mg-O distances

greater than 4.0 Å as it can be observed in Figure 8A. On the contrary, starting from a fully magnesium-substituted Posner cluster and increasing the number of  $\text{Ca}^{2+}$ , a loss of the structure for Ca-O distances greater than 4.0 Å occurs (see Figure 8B). This behavior can be rationalized considering the higher hardness of  $\text{Mg}^{2+}$  ion than  $\text{Ca}^{2+}$  ion, as expected. To further ascertain the stabilizing character of magnesium, the structure of the crystalline phase which AMCP particles tend to convert to (brushite, see XRD, FTIR and SEM results previously discussed) was considered, and it was re-optimized upon substitution of all calcium atoms with magnesium atoms (Brushite-Mg). Keeping fix the cell parameters, the comparison between the atomic positions in the two optimized crystals confirms these findings, as can be appreciated in Table 4 and in Figure 8C. All the intermolecular distances involving the  $\text{Mg}^{2+}$  ion and the first neighbors are shorter than those corresponding in the calcium crystal, as it can be observed in Table 4. The shortening in the distances is in agreement with the optimization calculations on the Posner's clusters.



**Figure 8.** Distribution of the Mg-O interactions (A) and Ca-O interactions (B) by increasing the number of Mg atoms (A) or Ca atoms (B). (C) Identification of atoms in Brushite-Mg structure as in Table 4.

**Table 4.** Distances between metal cation and first neighbors in optimized Brushite-Mg and Brushite crystal structures. The identification of the atoms refers to Figure 8C.

|                   | Distance (Å) - Brushite-Mg | Distance (Å) - Brushite |
|-------------------|----------------------------|-------------------------|
| $M^{2+}-O_1 (O)$  | 2.03                       | 2.37                    |
| $M^{2+}-O_2 (OH)$ | 2.08                       | 2.38                    |
| $M^{2+}-O_3 (O)$  | 2.04                       | 2.51                    |
| $M^{2+}-O_4 (OH)$ | 2.14                       | 2.51                    |
| $M^{2+}-O' (O)$   | 2.36                       | 2.45                    |

|                   |      |      |
|-------------------|------|------|
| $M^{2+}-O''(OH)$  | 2.57 | 2.67 |
| $M^{2+}-M^{2+''}$ | 3.91 | 4.04 |

In conclusion, the simulations well agree with the experimental results, as the comparison of the crystallization temperature and the interaction energies in Figure 7 both indicate a stabilizing effect of  $Mg^{2+}$  when it is included in the structure of ACP.

#### 4 Conclusions

In this work, the effect of Mg-substitution on the features of ACP nanoparticles is discussed. In biological organisms, Mg-substituted ACP nanoparticles are present in different regions and fulfill some important functions [2]. For this reason, understanding the role of  $Mg^{2+}$  in ACP structure and stability is important both to gain insights into the biological mechanisms as well as to prepare nanoparticles with defined composition to reproduce and study *in vitro* biological processes. In this context, some reports in the literature describe the preparation of AMCPs with different Ca/Mg ratio [24,38] and their stability in simulated body fluids [39,40]. Nevertheless, the features of the amorphous phases and the effect of  $Mg^{2+}$  in the amorphous cluster are poorly investigated.

In this paper a systematic analysis on this topic was conducted by performing 15 different syntheses in which the concentrations of the reacting solutions were varied to test 5 different Ca/Mg ratios (0.4, 0.7, 1, 2 and 3), each at 3 different  $Mg^{2+}$  concentrations (10, 50 and 100 mM). The precipitated AMCP nanoparticles were characterized with a multi-technique approach which allowed for the assessment of the structural features. We succeeded in identifying the best synthetic conditions to obtain pure amorphous nanoparticles and to prevent the formation of crystalline brushite. Despite XRD, FT-IR and SEM analyses did not show significant differences among the prepared particles, the analysis of their elemental composition revealed a variable Ca/Mg ratio in the particles, which was correlated to the synthetic conditions. Most importantly, calorimetric analyses demonstrated that the amount of

Mg<sup>2+</sup> in the particles has a key importance in delaying the thermal crystallization of AMCP, suggesting a stabilizing effect of this ion when included in ACP structure. These results are strengthened by computational analyses in which different Posner's-like clusters containing an increasing amount of Mg were modelled. The computed Mg-O and Ca-O distances in the clusters, as well as the interaction energies, confirm a stabilization of the cluster with the increase of Mg atoms. The presented results could be useful for researchers working on inorganic colloids and in particular on amorphous phosphates, as they provide a simple but effective strategy to tune the composition and thermal stability of AMCP nanoparticles; furthermore, from a fundamental perspective, they contribute to shed light on the effect of Mg<sup>2+</sup> substitution in ACP-based systems. These nanoparticles could be of interest for biomedical applications, for instance in the treatment of dentin hypersensitivity [41,42], or to reproduce and study the endogenous nanoparticles present in the small intestine [43].

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