Formation and Transformation of Calcium Phosphate Phases under Biologically Relevant Conditions: Experiments and Modelling.

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Abstract

The experimental data on calcium phosphates formation were collected in dilute solution at constant pH (7.40) and temperature (37.0 °C) at different levels of ionic strength (IS). The evolution of the solid phase formation is described in detail using a thermodynamic-kinetic model. The thermodynamic model takes into account all relevant chemical species as well as Posner's clusters; the kinetic model, based on the discretized population balance approach, accounts for the solid formation from solution. The experimental data are consistent with an initial formation of dicalcium phosphate dihydrate (DCPD, brushite), which dominates the nucleation rate, and its rapid transformation into octacalcium phosphate (OCP) or hydroxyapatite (HA), which dominates the growth rate. Depending on the experimental conditions and, including the influence of the IS level, OCP may be further transformed into apatite. The classical nucleation theory is able to describe the experimental results very well and the solid phase growth is limited by the diffusion of Ca^{2+} ions. The precipitation pathway described by a complete thermodynamic-kinetic model is expected to contribute to the understating of the *in vivo* osteogenesis.

Keywords: calcium phosphates, precipitation kinetics, modelling, classical nucleation, diffusion-limited growth.

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1. Introduction

Calcium phosphates are largely studied in many fields, such as chemistry, material science, and geology, as well as in biomedical applications, in which they are used to coat metallic implants [1] and as biocements [2]. Calcium phosphates include a large group of biomaterials [3]. Among them, the apatite, $\text{Ca}_{10}(\text{PO}_4)_6\text{X}_2$, where X corresponds to OH in hydroxyapatite (HA), and those related to apatite-like structures, such as the octacalcium phosphate, $\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$ (OCP), are the most relevant in natural bone formation. Nowadays, OCP is considered the *in vivo* precursor of the thermodynamically stable calcium phosphate form, HA, which is the main mineral constituent of bone and teeth. The role of OCP in the osteogenesis was the object of debate, since Brown *et al.* [4-7] suggested that this mineral is the intermediary phase in the biological calcification process, being present as a transient phase in hard tissues. The authors identified several lines of evidence to support their conclusions. One of the most relevant observations was related to the peculiar platy shape morphology of OCP, which is conserved after *in vitro* hydrolysis to HA crystals whereas needle-like morphology would be expected if HA had precipitated directly from solution [8]. OCP surrounded by HA was detected by HRTEM (High-Resolution Transmission Electron Microscopy) in the dentin of aborted fetuses [9] and the hypothesis that OCP is the crystalline phase occurring in young bones got more and more support. *In vivo* experiments have shown that OCP is rapidly converted to HA, *i.e.*, within 7-10 days after implantation and, interestingly, the rate of new bone formation on apatite converted from OCP was faster than that on both calcium-deficient HA and stoichiometric HA implanted directly [10, 11, 12]. The effect of calcium phosphates on the osteoblastic activity and bone regeneration, with particular emphasis on OCP activity, has been recently reviewed [13] concluding that, *in vivo*, OCP seems more bioactive than other calcium phosphate phases.

The synthesis of OCP in solution and its bioactivity is relatively well reported in the literature [13]. According to the opinion of present authors, the most relevant studies on OCP and HA were carried out by Nancollas *et al.* [14, 15] and by Iijima *et al.* [16]; in these studies the constant composition approach was applied. Such a method is particularly suitable to study the details of the precipitation pathway.

The formation mechanism of calcium phosphate – and, in general, that of all sparsely soluble

inorganic salts – is also a matter of debate. In the particular case of calcium phosphate, the presence of nanometric size clusters (Posner's clusters [17]), and their involvement in the solid formation mechanism has been suggested [18-22]. Posner's clusters were experimentally detected by cryo-HRTEM (cryogenic HRTEM) [23] and AFM (Atomic Force Microscopy) [24] and their involvement in the solid growth process was proposed, paving the way to the so-called non-classical precipitation theory [25], in which nanometer-size building blocks are considered as the growth unit of the solid. A similar debate is currently ongoing on CaCO_3 whereas the most recent literature is in favour of the classical view, in which the solid grows by incorporation of ions, while larger clusters – if they are present – act as spectator species [26, 27].

Despite a considerable number of papers on calcium phosphate precipitation, to the best of our knowledge, a complete mathematical description based on a thermodynamic-kinetic model, which is able to simultaneously solve the chemical speciation and the details of the nucleation and growth pathway, is still missing [28]. In a comprehensive paper [29], Wang L. and Nancollas G.H. highlighted some open key questions about the formation mechanism of calcium phosphates. In particular, they focused their studies on the driving force for the solid formation, stating, “*Nucleation and growth may follow different mechanisms across the continuum of driving force*”.

In this paper, we address some of the aforementioned open questions, shedding some light on the precipitation mechanism. Here, the applied approach is similar to that followed in the study of the amorphous calcium carbonate (ACC) precipitation pathway [26], and we demonstrate that, in our experimental conditions, the experimental data are fully consistent with a classical precipitation pathway. The mechanism involves the initial formation of a solid phase with $\text{Ca/P} = 1$ and its rapid transformation into OCP or HA. Such a pathway was already postulated by Francis and Webb [30]; here, we present the detailed mathematical description. Moreover, the solid formation driving force changes during the precipitation pathway in a rather complex manner. The described mechanism may be replicated, under similar physicochemical conditions, in biological systems during osteogenesis or pathological mineralization and can contribute to the understanding of biomineralization-biodesmineralization processes. Furthermore, the knowledge of the solid formation driving force will allow the *in vitro* controlled deposition of calcium phosphate bioceramics on implants and prostheses.

2. Material, methods, and model

The precipitation reaction was studied using a controlled composition approach and potentiometric titration method [26]. Specifically, the system saturation level was slowly increased by means of CaCl_2 addition in a $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ solution. Experimental data were collected at a fixed temperature, initial concentration of phosphate buffer, and pH ($T = 37.0\text{ }^\circ\text{C} \pm 0.2\text{ }^\circ\text{C}$, total P = 10.0 mmol kg^{-1} , pH = 7.40 ± 0.03). The pH was monitored and adjusted at the pre-set value via counter-titration with NaOH or HCl solutions. The precipitation was studied at different ionic strength (IS), which is controlled by the addition of NaCl in the precursor solutions. The first series of experiments were carried out at low IS (0.024-0.017, *i.e.*, without the addition of NaCl into the initial solutions). The second series at high IS (0.149-0.088, *i.e.*, adding NaCl into the phosphate buffer only). The third series at constant IS (0.154, *i.e.*, adding NaCl in every solution used during the precipitation). In the first and second series, hereafter called L-IS and H-IS, during the precipitation the IS drifted, mainly because of the dilution due to the addition of CaCl_2 and NaOH solutions; in the third series, hereafter named C-IS, the IS was maintained at a constant level, *i.e.* the physiologically relevant conditions. Each series consists of at least 3 repetitions. The precipitated solids were separated from the solution by centrifugation and thus, washed with milliQ water, acetone and stored dry. For morphological studies, the solid was re-dispersed in isopropanol. Additional details on the experimental protocol are reported as Supporting Information (Section S1).

The mathematical model is developed in FORTRAN and it is composed of two main packages: the thermodynamic speciation solver and the solid kinetic solver. Both packages are cyclically called until self-consistency at every time step is reached [26, 31].

The thermodynamic model is based on the chemical equations reported in Supporting Information (Appendix-B). In particular, 22 chemical species are considered: 19 aqueous species and three solid phases namely dicalcium phosphate dihydrate (DCPD), OCP, and HA. Additional to the system of 14 equations associated with the chemical equations, two mass balance equations for total Ca and total P are included. Thus, the problem can be solved if six quantities are known, namely the pH value, total Ca, total P, and the quantities of the three solid phases. The amounts of solid phases are computed by the kinetic solver whereas the other three quantities are given. The system of 16 equations is analytically reduced to 2 equations only,

which are efficiently numerically solved at every call of the speciation package (SI, Appendix-B, Eqs. 17-18).

The kinetic package includes the equations for primary nucleation, secondary nucleation and diffusion limited growth. The evolution of the solid particles is computed by means of a discretized population balance approach, where the continuum of size is divided into classes. Each class is associated with a differential equation and the system of ordinary differential equations is solved at every time step. The details of the population balance approach are described elsewhere [31]. At this level, the kinetic solver is used to compute the amount of solid phase formed, where the particles are considered as spheres. Since the experimental evidence shows that particles are not equiaxed, no information on particle size distribution can be argued, unless a defined particle thickness is set. This limitation is due to the applied population balance approach being monovariate, thus only one internal parameter (*i.e.*, the particle equivalent diameter) can be considered. Within this constraint, the applied approach is able to correctly calculate at least the first four moments of the particles population [31]. The implication of the assumed particle shape is discussed later.

Because of the addition of CaCl_2 into the reactor, both the activity of $\text{Ca}^{2+}_{(\text{aq})}$ and pH change. In order to attain iso-pH conditions, a diluted NaOH solution was added into the reactor. Thus, two independent datasets were collected against time: (i) the activity of $\text{Ca}^{2+}_{(\text{aq})}$ and (ii) the amount of NaOH added into the reactor. It is worth mentioning that the needed amount of NaOH is due to the formation of calcium-phosphate species such as $\text{CaPO}_4^{-}_{(\text{aq})}$, $\text{CaHPO}_4_{(\text{aq})}$, $\text{CaH}_2\text{PO}_4^{+}_{(\text{aq})}$, Posner's clusters, and the solid phases DCPD, OCP, and HA. The amount of NaOH added can be analytically calculated, provided that the formation constants of the aforementioned species are known. Among them, only the formation constant of Posner's clusters is unknown and can be regressed from the experimental data. The considered parameters in the model are the surface energy for primary and secondary nucleation, the stoichiometry and formation constant for the Posner's clusters, the stoichiometry of the solid phases formed and their solubility, the transformation rate from OCP to HA, and the diffusion coefficient of the chemical species involved in the molecular growth. At every set of parameters, the calculated evolution of $\text{Ca}^{2+}_{(\text{aq})}$ activity and the amount of NaOH added into the reactor against time are compared with the respective experimental curves. The model is considered appropriate when the calculated curves are able to correctly describe the experimental data in the full timeframe and either the regressed

parameters are consistent with the literature values (if available) or they correspond to reasonable values.

The collected powders were fully characterised. The chemical analyses were performed by ICP-MS (Agilent 7700x) (Inductively Coupled Plasma - Mass Spectrometry), after digestion of the samples and dilution in Milli-Q water, and using external standard calibration.

Synchrotron X-ray Powder Diffraction (SXRPD) patterns were collected at the X04SA-Materials Science beamline, Swiss Light Source (SLS), Paul Scherrer Institut (PSI). The X-ray beam energy was set to 22 keV, and the powders were confined in 0.1 mm rotating glass capillaries in Debye-Scherrer geometry.

Morphology and composition of the samples were analyzed by a scanning electron microscopy (Zeiss NVision40,) equipped with energy-dispersive spectroscopy (EDX, Oxford). HRTEM and high-angle annular dark field (HAADF) scanning transmission electron microscopy (STEM) images were acquired with a Talos F200X (FEI) microscope being operated at an acceleration voltage of 200 kV. The powders for SEM and STEM characterization were dispersed in isopropanol and then deposited onto carbon-coated or holey carbon-coated copper grids, allowing the solvent to evaporate in air.

The infrared spectra were collected in the ATR-IR mode using a Vertex 80V spectrometer (Bruker) equipped with a Platinum ATR unit by accumulating 100 interferograms at 4 cm⁻¹ resolution.

The Raman spectra were acquired on dried powder samples using a Raman spectrometer (RamanRXN1 Analyzer supplied with Invictus™ 785-nm laser; Kaiser Optical Systems) equipped with a CCD camera (1024x256 EEC MPP Type, Kaiser Optical Systems) with collimated incident radiation and a probe size of 2 mm. Spectra were collected at ambient conditions by averaging 300 scans with 2 s of exposure time at 2 cm⁻¹ spectral resolution, and at an average power of 300 mW.

3. Results

Figure 1 shows the experimental and the calculated results for the Ca^{2+} activity (A, C) and the amount of NaOH (B, D) added into the system for the series H-IS and L-IS. The results obtained for H-IS are almost identical to those for C-IS (Supporting Information). The experimental data collected for the series H-IS and C-IS showed a high reproducibility, whereas the experimental data for the series L-IS were much more scattered and several repetitions were required in order to identify an appropriate variability range.

The experimental and calculated curves are reported as black and coloured lines, respectively. In all figures, zero on the time axis corresponds to the addition start of calcium chloride solution into the reactor. The activity of Ca^{2+} in solution rises almost linearly and proportionally to CaCl_2 added into the system (Ca_T , Supporting Information) up to a maximum; it decreases down to a minimum following a (inversed) sigmoidal shaped curve, and finally an almost constant value is reached. The slightly positive slope at the plateau is due to the depletion of PO_4^{3-} ions from solution and the speciation equilibrium with the formed solid. In parallel, NaOH is added into the system and a three-linear-segments-shaped signal is acquired; the steepest segment corresponds to the rapid solid formation whereas the first and last segments identify the two equilibrium stages before precipitation and in the presence of the solid, respectively.

The maximum Ca^{2+} activity in Figure 1A (H-IS) is higher than that in Figure 1C (L-IS), but the same initial slope and almost the same final plateau levels are attained. Since the experiments refer to the different IS levels, they can be compared in terms of Ca^{2+} activity, provided that the appropriate activity coefficient calculation model is applied [26].

The chemical speciation for Ca and P species is reported in Figure S2 (Supporting Information). The predominant Ca species in solution are Ca^{2+} , CaHPO_4^0 , CaPO_4^- and Posner's clusters. The stoichiometry of the latter and their stability constants are regressed from the experimental data. In the model, every stoichiometry for Posner's clusters is possible, such as $\text{Ca}_x(\text{PO}_4)_y\text{OH}_n\text{H}_m\text{Na}_i\text{Cl}_j^{k+}$. The best fitting is obtained considering the stoichiometry as $\text{Ca}_6(\text{PO}_4)_4$ (6:4 clusters) for L-IS series and $\text{Ca}_9(\text{PO}_4)_6(\text{OH})_4^{4-}$ (9:6 clusters) for H-IS, and C-IS. The estimated stability constant (conditioned, *i.e.*, valid in the specified IS) of these clusters resulted to be 3.0×10^{46} for L-IS, and $2.5\text{-}4.0 \times 10^{95}$ for H-IS, and C-IS.

The predominant P species are HPO_4^{2-} , H_2PO_4^- , and NaHPO_4^- . The concentrations of pyrophosphate-related ions (*e.g.*, $\text{HP}_2\text{O}_7^{3-}$, $\approx 10^{-6} \text{ mol L}^{-1}$) is at least three orders of magnitude lower than those related to orthophosphate ions (*e.g.*, NaHPO_4^- , $\approx 10^{-3} \text{ mol L}^{-1}$) but their consideration into the speciation model is required to attain a relative mass balance error at the limit of the computational error ($< 5 \times 10^{-10}$) and an overall charge unbalance lower than $5 \times 10^{-17} \text{ mol kg}^{-1}$.

For series L-IS and H-IS, the concentration of Na^+ and Cl^- decrease (as well as IS) because of the dilution of the system due to the addition of CaCl_2 and NaOH . For the series C-IS, Na^+ , Cl^- , and IS are constant during the entire experimental timeframe (Figure S3).

Figure 2 reports on the calculated primary and secondary nucleation rate and the critical size for primary and secondary nucleation, in the supersaturation range where such events occur, for H-IS series.

The critical supersaturation value from the regressed model was found to be 2.53 (2.28) for the H-IS (C-IS) series, and 3.45 for the L-IS series (Figure S4). As discussed later, the critical supersaturation refers to the solid phase responsible for nucleation, *i.e.* DCPD. It is worth mentioning that in this paper supersaturation (S) is defined as IAP/K_{sp} , (where IAP is the Ion Activity Product calculated from the speciation model), without any exponential normalization for the solid phase stoichiometry, according to the classical nucleation theory described by Nielsen [32].

Since the nucleation is governed by the formation of DCPD (see section 4), the calculated surface energy for primary (γ) and secondary (γ_{eq}) nucleation, refers to that phase and not to OCP (or HA) which is the final product of the precipitation. The secondary nucleation takes into account for the generation of new primary nuclei on the surface of the already formed solid [26, 31], thus for the self-catalytic behaviours of the precipitation. Among the three series of experiments, the calculated surface energy almost scaled linearly with the IS in the nucleation range (Figure S8, Supporting Information). For instance γ resulted to be 42.5 mJ m^{-2} , 35.5 mJ m^{-2} , and 32.8 mJ m^{-2} for C-IS (IS=0.154), H-IS (IS=0.125), and L-IS (IS=0.022), respectively. The adhesion energy (β) [26, 31] is estimated to be $69 \pm 1 \%$ of the maximum theoretical value (2γ) for all the experiments. The estimated size-averaged maximum linear growth rate during the fast growth stage (point *d*, Figure 1) is $6.5 \times 10^{-9} \text{ m s}^{-1}$ ($7.1 \times 10^{-9} \text{ m s}^{-1}$) for H-IS (C-IS) series and 5.6

$\times 10^{-9} \text{ m s}^{-1}$ for the L-IS series. These values are reasonable for sparsely soluble salts. In the following stage (*f-g*, Figure 1) the growth is limited by the Ca^{2+} addition into the reactor and the estimated size-averaged linear growth rate ranges (for all experiments) from $4 \times 10^{-11} \text{ m s}^{-1}$ (in *f*, Figure 1) to $2 \times 10^{-11} \text{ m s}^{-1}$ (in *g*).

The diffusion coefficient is estimated to be $3.1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ which is substantially lower than that calculated for Ca^{2+} ions ($1.08 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$) at the experimental condition of temperature (37 °C) and dynamic viscosity ($6.85 \times 10^{-4} \text{ kg m}^{-1} \text{ s}^{-1}$). The precipitated solids were characterized by IR and Raman spectroscopy, high-resolution X-ray diffraction (XRD), electron microscopy, and chemical analysis, and both OCP and HA were detected.

The clearest evidence for phase composition is given by the collected high-resolution X-ray diffraction patterns (Figure 3) because of the intense characteristic peak (100) of OCP at a low angle.

As reported in the literature, maturation time, pH value, temperature, and the way of addition of reactants influences the precipitated solid phase [33, 34]. These results were confirmed by our experiments. In particular, in the series H-IS, the solid collected for short ageing time (450 min) contains a high amount of OCP but it evolves towards HA if calcium solution addition is suspended and the solid is aged for several hours (Figure 3, pattern H-IS, aged). Nevertheless, in the series C-IS, OCP seems more stable and the evolution toward HA was not clearly detected during the experimental time frame. Nevertheless, both OCP and HA phases are present in the collected solid phase. Here, Rietveld refinement is used to evaluate the lattice parameters of the solid phases but the quantitative evolution of the solid composition is questionable since not all the peak intensities can be properly fitted, most probably because of the particle shape and incertitude on the solid phase stoichiometry. For the L-IS series, the obtained solid phase was HA even for short ageing time. Thus, a trend between IS and OCP stabilization can be speculated.

The unit cell parameters obtained by Rietveld refinement for the L-IS series samples collected for both short (250 min) and long (600 min) maturation time (Figure 3) were $a = 9.451 \text{ \AA} \pm 0.002 \text{ \AA}$ and $c = 6.875 \text{ \AA} \pm 0.002 \text{ \AA}$. Similar values are reported for freshly precipitated HA [35]. Instead, for the samples of the series H-IS and C-IS (Figure 3), the diffraction patterns show clearly the intense characteristic peak (100) of OCP at low angle. The refined OCP cell parameters are $19.713 \text{ \AA} \pm 0.002 \text{ \AA}$, $9.525 \text{ \AA} \pm 0.002 \text{ \AA}$, $6.840 \text{ \AA} \pm 0.002 \text{ \AA}$, $90.12^\circ \pm 0.01^\circ$,

$92.53^\circ \pm 0.01^\circ$, and $108.30^\circ \pm 0.01^\circ$ for a , b , c , α , β , and γ , respectively.

Ca/P molar ratio measured by chemical analysis was 1.43 and 1.50 for L-IS and H-IS (C-IS) experiments, respectively. These values are below the 1.67 expected in case of crystalline apatite and above 1.33 expected for OCP phase, which is in agreement with the co-existence of both phases.

Figure 4 shows representative images of C-IS and L-IS series samples, corresponding to the diffractograms of Figure 3.

The sample of the series C-IS (Figure 4B), in which the OCP phase is present in a relevant amount, show well-developed platy-like crystals of (1-3) μm and (5-10) nm thickness, which are commonly associated to the OCP phase [3], together with needle-like crystals, generally associated to HA phase [36]. The sample of L-IS series (Figure 4A) shows a less developed structure, with smaller crystals when compare to the other series, together with a sponge-like structure, which is commonly present in HA samples [37].

TEM micrographs allow further insight of the nanostructure (Figure 5). First of all, the thin crystals are very sensitive to the electron beam and artefacts can be easily generated during the observation. Secondly, the fringes associated with the most intense OCP peak (1.87 nm) were not detected since most platy crystallites were preferentially lying with their (100) plane on the carbon support. Difficulties to identify such a planar distance were reported in the literature [38] even because the material is very sensitive to electron bombardment and rapidly recrystallized [39]. Thirdly, only crystalline particles were detected. Figure 5 (C, D) shows the large and thin OCP crystals whereas smaller acicular crystallites were detected in the case of HA (Figure 5 A, B). However, few large platy-like crystals where detected in the HA samples as well, probably as a memory of an OCP transient phase.

Raman and IR spectroscopies confirmed the co-existence of both phases, in agreement with diffraction results (Section S3, Supporting Information) and the absence of carbonate contamination.

4. Discussion

Several combinations of nucleation and growth models were considered in the code, but only one of them was able to describe all the experimental results exhaustively, with full agreement with the literature and with the characterizations of the solid. In particular, every attempt to simulate the direct nucleation of OCP or HA fails, resulting in inconsistent nucleation and growth parameters, supersaturation level, and solubility of the solid phases. Instead, if only a solid phase with $\text{Ca/P} = 1$ is considered, the model is able to describe the evolution of the $\text{Ca}^{2+}_{(\text{aq})}$, but it is not able to cope with the amount of NaOH added into the reactor for iso-pH condition, nor, with the experimental results about the crystal phase collected and its solubility.

The best description of the precipitation pathways is obtained by hypothesizing that primary and secondary nucleation [26, 31] are driven by the formation of DCPD, which is rapidly transformed to OCP (or HA depending on the experimental conditions). Growth is diffusion limited, under the driving force of the solubility of OCP (or HA). According to this assumption, every detail of the precipitation is described, and every parameter of the model is completely consistent with the literature. The calculated critical supersaturation, which triggers primary nucleation, is relatively low since DCPD is accounted as the nucleating phase and has a much higher solubility than OCP or HA. The considered DCPD solubility is 2.19×10^{-7} in all experimental conditions [40]. Otherwise, if OCP (or HA) is considered as nucleating phase, the critical supersaturation value would correspond to about 4×10^3 (or 2×10^4 , respectively), resulting in inconsistent low surface energy values associated to the corresponding solid phases and an unreasonably high nucleation rate. According to our model, the critical supersaturation with respect to DCPD is in the range of 2.5-3.5, which is in line with values reported for other sparsely soluble inorganic compounds. The involvement of an intermediate solid phase in the formation of OCP was already postulated being amorphous calcium phosphate, ACP [29], or DCPD [30, 41]. Despite the absence of a long-range periodic order, ACP appears to have a relatively constant composition, with a Ca/P of about 1.5 [42, 43] or as low as 1.15 [44]; the latter is very unstable and it rapidly re-crystallizes into crystalline DCPD. Our experimental evidence is consistent with an intermediate nucleating phase with a Ca/P ratio = 1, but, to date, we cannot speculate if it is crystalline or not. Both DCPD and dicalcium phosphate anhydrous DCPA (monetite) are plausible: DCPA is thermodynamically slightly more stable than DCPD,

but the hydrated form is considered the kinetic product, having lower surface energy [45], and its formation is consistent with the Ostwald's empirical rule of states.

In the proposed precipitation pathway, OCP can be considered as the natural intermediate between DCPD and HA since it is composed of apatitic and hydrated layers. The apatitic layers are structurally related to HA whereas the hydrated layers are compositionally related to DCPD.

An additional line of evidence to support the involvement of DCPD instead of ACP is given by the fact that in the nucleation conditions the system is supersaturated with respect to DCPD but undersaturated with respect to ACP [34].

Figure 6 represents the chemical potential plots of the experimental data obtained by Iijima *et al.* [3, 34], and the experimental data of the current work (Figures 7A and 7B for H-IS and L-IS, respectively).

In Iijima's work (Figure 6), the initial conditions are indicated as shaded green points, and the system evolves towards the formation of OCP or HA (blue and pink shaded points, respectively) depending on the experimental conditions. In parallel, Figure 7A represents the precipitation pathway for the H-IS series where OCP is obtained. At position *a* in Figure 7A the addition of Ca^{2+} starts and the system is immediately supersaturated with respect to HA. The addition of CaCl_2 proceeds and from position *c* the system is supersaturated even with respect to DCPD. Around point *d* nucleation occurs when the system remains undersaturated with respect to ACP. Thus, ACP precipitation does not occur. At point *e* the system becomes undersaturated with respect to DCPD but still supersaturated with respect to both OCP and HA. According to our calculation, at this point primary and secondary nucleation events are already negligible (Figure 2), whereas the growth rate is still close to its maximum rate. Plausibly, DCPD is rapidly dehydrated and re-crystallized in a more stable solid phase (also Figure 2B, point *e*). The latter becomes the predominant phase in the section *f* and in equilibrium with the solution in section *g*. The same points (*a-g*) are represented in Figure 1A. That solubility equilibrium does not correspond to the literature value of OCP but, in the diagram of Figure 7, a line parallel to that of OCP equilibrium is identified, *i.e.* the precipitated solid phase has the Ca/P ratio of about 1.33. Similar results are reported in Figure 6 (blue-shaded points). The presence of OCP in the collected solid phase was confirmed by XRD (Figure 3).

Figure 7B reports on the precipitation pathway for the L-IS series where HA is obtained (XRD,

Figure 3) and the slope in the region where the equilibrium with the solid phase is attained, is equivalent to that of HA. Again, similar results are reported in Figure 6, where the pink shaded area identifies the formation of a solid phase with a crystal structure and Ca/P ratio of HA. The obtained solid phase in Figure 6 was driven by different Ca/P ratio in the initial solution (shaded green points) and the Ca^{2+} addition rate into the reactor. It was also reported that OCP is transformed to HA as soon as Ca^{2+} addition is suspended; stirring has a relevant influence as well [3, 34]. According to our experimental results, IS influences the selection of the formed solid phase. All these evidences indicate that the solubility of the solid phases obtained in such *in vitro* conditions is higher than those reported for OCP and HA. In particular, the solubility constant of OCP (as $\text{Ca}_4(\text{PO}_4)_3\text{H}$) corresponds to 2×10^{-48} and that for HA (as $\text{Ca}_5(\text{PO}_4)_3\text{OH}$) corresponds to 2×10^{-51} . In literature a wide range of solubility is reported for apatitic calcium phosphates, ranging from $10^{-42.5}$ to 10^{-66} [46-52]. This considerable variability is ascribed to the possible incorporation of foreign ions into the structure. Our experiments are conducted in a carbonate-free environment (and spectroscopic evidences confirm the absence of carbonates in the structure), but the incorporation of chloride or sodium ions is plausible, even if not EDX detectable (Figure S7). Similarly, the experimental results of Figure 6 are collected in a carbonate- and chloride-free environment and a relatively high solubility for the formed solid phases is reported, too. The solubility values for OCP and HA are very close to each other and their coexistence is plausible. Our diffraction and spectroscopic characterizations also confirm the coexistence of both phases (Figure 3 and S6). Moreover, the structural similarities between OCP and HA allow the epitaxial growth of HA on OCP [53] (see refined lattice parameters b and c , Section 3), and further they suggest that OCP is a plausible precursor of HA in the early stage of bone and teeth formation.

The modelling results are consistent with the solid phase obtained. If OCP is set at solid phase formed for L-IS series – as well as if HA is set as solid phase for H-IS or C-IS – the experimental data for Ca^{2+} activity might be somehow reproduced, but the amount of NaOH needed is mostly inconsistent with the experimental results. In the H-IS series, a slow transformation of OCP to HA is also included into the model, using an empirical equation (SI, Appendix-A, Eq. S13). Thus, 300 minutes after the nucleation stage, the model calculates that about 20 mol % of OCP is transformed to HA. This transformation takes into account the experimental slope of the NaOH added volume against time in the segment f - g in Figure 1B. This transformation is also supported

by XRD results, even if diffraction patterns show a higher amount of HA with respect to the model prediction. Nevertheless, since a single solid phase can be included into the population balance modelling, only an empirical equation was considered to take into account the solid-solid OCP-HA transformation.

According to the modelling results, the existence of DCPD as transient phase is plausible but its direct identification is very difficult. This intermediate phase may exist only in a limited time frame (few minutes, from point d to point e in figure 1) and at very low concentration since only nuclei are produced. They become rapidly undersaturated and, according to the model, have to disappear in e. Therefore, DCPD should not present in the final product. Even in case DCPD does not recrystallize towards OCP or HA, its fractional amount is too low to be detected (<0.1 wt %).

In the C-IS series, no transformation towards HA was introduced to fit the experimental data, in agreement with diffraction results, where a high amount of OCP is detected. It is also worth mentioning that the time-resolved experimental data collected in solution are used to calculate the solid phase composition at the end of the precipitation, but the characterized solid was collected, washed and dried: some modification of the solid phase due to these operations cannot be excluded. Thus, honestly speaking, the quantitative analysis obtained by Rietveld refinement on the OCP/HA ratio evaluated on the collected solid phases, as well as the other characterizations on the solids, can only partially be called as an experimental validation of the modeling results. The final model validation can be obtained by *in-situ* time-resolved characterization of the solid in the section *d-f* of Figure 7, which is the objective of our current research. To date only the trend in the experimental result can be outlined. In the L-IS, HA is obtained even for short ageing times; in H-IS, OCP is obtained, and it is transformed to HA during the experimental time frame of some hours; in C-IS, OCP appears stable during the experimental timeframe and in coexistence with HA.

Considering that temperature, pH value, phosphate concentration, as well as Ca^{2+} activity in the range (*f-g*) (Figure 1A, C, S1) are constant among all the experiments and the fact that IS has an influence, it could be concluded that the activity of phosphate ions plays a role, being the activity coefficient of such species influenced by the IS. The activity coefficient for PO_4^{3-} corresponds to 0.06, 0.06-0.10, and 0.25-0.30, for C-IS, H-IS and L-IS, respectively. Thus, according to this interpretation, it can be speculated that the activity of phosphate ions – and their local speciation

– is determining the osteogenesis thermodynamics whereas Ca^{2+} ions diffusion is controlling the osteogenesis kinetics.

Beside the solid phase obtained, the modelling results can suggest a possible growth mechanism, and some indication about the nature of the building unit deduced. In the region (*f-g*), the solid growth is limited by Ca^{2+} addition [26], thus under thermodynamic control. Instead, in the intervals (*d-f*) of Figures 7A,B and Figure 1A,C, the growth is limited by the precipitation kinetics (fast growth interval), and these data are useful in the evaluation of the growth mechanism. In the code, several diffusion-limited growth models are implemented, and the local surface equilibrium condition is assumed. The best results are obtained when assuming that the growth is limited by Ca^{2+} ion diffusion. In spite of that, the diffusion coefficient obtained is lower than that estimated for Ca^{2+} ions at these experimental conditions of temperature and viscosity. This calculation might be interpreted in various ways:

- i. A low diffusion coefficient can be associated with species of a size substantially larger than ions, such as clusters. Applying the Stoke-Einstein equation to the aforementioned diffusion coefficient, the estimated hydrodynamic diameter resulted to be about 2.2 nm, which might be in agreement with Posner's cluster with a diameter of about 1 nm. Moreover, our thermodynamic calculations are consistent with the presence of Posner's clusters in solution. Nevertheless, their concentration ranges between about $2 \times 10^{-5} \text{ mol kg}^{-1}$ close to the nucleation zone (Figure 1A, *d*) and as little as to $(10^{-7} - 10^{-8}) \text{ mol kg}^{-1}$ in (*f*). In the same regions, the concentration of Ca^{2+} ions or CaHPO_4 ion pair is about 1 and 3 order of magnitudes higher, respectively, than that of Posner's clusters. Thus, it seems plausible that the nature of growth unit changes during the precipitation and the involvement of Posner's clusters in the initial fast growth rate stage cannot be excluded. However, such events are taken into account in the presented model and considered as secondary nucleation events and not as growth events. The calculated critical size for primary and secondary nucleation (Figure 2B) is close to the estimated hydrodynamic diameter of Posner's clusters and nucleation occurs in the zone where they are at maximum concentration. Thus, the involvement of Posner's clusters in the primary and secondary nucleation stages by dehydration and incorporation of PO_4^{3-} ions seems plausible;

- ii. The diffusion of PO_4^{3-} ions from solution might play a role in the growth mechanism, resulting in a reduced effective diffusion coefficient. In the model, the surface concentration of PO_4^{3-} ions is considered equivalent to the value in solution, *e.g.*, far from the growing surface. This approximation might be justified because in the experiments the concentration of phosphate buffer is three orders of magnitude higher than that of Ca^{2+} ions;
- iii. The particles are strongly anisotropic (Figures 4, 5): the incorporation of building units is possible only along a particular direction thus not all the solid surface is available for growth, resulting in a reduced growth rate, which is reflected in a reduced effective diffusion coefficient. In other words, an additional contribution due to the surface diffusion of Ca^{2+} ions needs to be considered. The influence of the particle shape on the growth mechanism has recently been studied for the calcium silicate system [54] where platy-shaped particles are produced: a similar approach is currently a future objective for our study for both the OCP and HA systems.

Very often, the involvement of clusters in the growth mechanism is claimed because they are experimentally detected both in solution and on the surface of growing crystals and because the calculated saturation level is too high to justify a slow growth rate. Nevertheless, since the precipitation pathway described here is controlled by a driving force that changes over time, the “true” supersaturation, *i.e.* that it needs to be considered to evaluate the driving force for solid formation, is always relatively low. For instance, in the H-IS series, at the beginning of the precipitation (a)-(e) the supersaturation is calculated with respect to DCPD ($S < 2.6$), but as soon as the point (e) is crossed, the supersaturation – which is now the driving force of the growth process in the range (f)-(g) – is calculated with respect to OCP ($\approx 10 < S < 1.05$) (Supporting Information, Figure S9). These changes induce a sort of physical discontinuity close to (e), which needs to be corrected in an even more sophisticated model, where the continuum of the driving force needs to be correlated to a continuum solid-solid DCPD-OCP transformation via dehydration. To be able to investigate such kinetic details, *in-situ* experimental data on the solid phase with a time resolution of the order of few seconds need to be collected; considering that, the concentration of the solid phase in (e) is in the range of (10-100) mg L^{-1} , such an experimental investigation is rather ambitious. So far, our most recent results, using synchrotron-

based small and wide angle X-ray scattering techniques, were just able to identify the presence of solid particles, but not their nature.

5. Conclusions

A quantitative mathematic model for the kinetic and thermodynamic description of the OCP and HA formation, first postulated by Francis and web in 1970, has been developed. The calculated solubility values for OCP and HA are close to each other, they are higher than those commonly assumed but consistent with the literature for solids prepared under similar conditions. In the thermodynamic package of the model, the only parameter regressed is the stability constant of Posner's clusters and their stoichiometry. The former allows the estimation of the Posner's clusters concentration during the entire solid formation process whereas the latter is in agreement with the literature and seems to be influenced by the ionic strength. It is plausible that Posner's clusters play a role in the nucleation stage where they may incorporate PO_4^{3-} ions generating primary and secondary nuclei of DCPD. The estimated surface energy from nucleation rate refers to the formation of DCPD, therefore cannot be associated to OCP or HA. After the nucleation stage, DCPD becomes rapidly undersaturated with respect to OCP and – in isotonic conditions – the latter crystallizes and grows limited by diffusion of Ca^{2+} ions. At lower IS, HA is formed instead. Growth rate is consistent with a Ca^{2+} diffusion-limited model even if the diffusion rate is lower than that expected for ions. This discrepancy can be justified by the platy-shaped particles and an additional surface diffusion contribution, determining a lower apparent diffusion coefficient. It turns out that all experimental data are consistent with a classical precipitation pathway, where primary and secondary nucleation events are considered, in which a transient phase is involved, and where the growth is mainly diffusion limited. Moreover, considering that pH, temperature, and phosphate buffer concentration are kept constant, and the same level of Ca^{2+} ion activity is attained at the end of the solid formation for all experiments irrespective of the IS level, it is argued that the selection of solid phase obtained is driven by the activity coefficient of PO_4^{3-} ions, which change by a factor of 3-5 among the experimental datasets: the higher the ionic strength, the lower the activity coefficient of PO_4^{3-} ions (i.e., the lower the activity of PO_4^{3-} ions at constant concentration), and the higher the stabilization of OCP with respect to HA. Moreover, the thermodynamic-kinetic model for solid calcium phosphates

formation is a useful tool to predict the appropriate experimental condition in order to promote the formation of calcium phosphate phases *in vitro* and can contribute toward the comprehension of the apatitic phases formation mechanism *in vivo*.

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Author contributions

A.Ca. carried out all the experimental data acquisition, characterization, data reduction, and analysis. She developed the analytical acquisition software, the preliminary thermodynamic calculations, and the adaptation of the computational code to the specific problem discussed in this paper. She wrote the paper and SI and generated all graphical outputs. Ch.L. contributed to provide the financial support. A.Ce. coordinated the diffraction data collection at the beamline. E.M. carried out the EM investigation and contributed to the micrographs, EDX, and ED interpretation. A.T. developed the modeling core code and coordinated the entire scientific activity. All co-authors entirely reviewed the manuscript and SI and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Figure 1 – Experimental (black) and calculated (red) data. (A,C) Ca^{2+} molal activity in solution, derived from the ISEs electrodes for H-IS and L-IS experiments, respectively; (B, D) volume of NaOH added into the system to keep iso-pH conditions for H-IS and L-IS experiments, respectively. The letters (a-g) in A and C are discussed in the text.

Figure 2 – Calculated kinetic data for H-IS series. (A) Primary (purple, $\cdot\cdot-$) and secondary (blue, $--$) nucleation rates; (B) critical nucleation size for primary homogeneous (purple, $\cdot\cdot-$) and “true” secondary nucleation (blue, $--$) at the DCPD supersaturation level (red line).

Figure 3 – Diffraction patterns, after background subtraction, collected on dry powders. From top to down: reference pattern for HA (ICSD 16742); reference pattern for OCP (ICSD 65347); samples of the series C-IS (which shows equivalent results to samples of the series H-IS collected for short ageing time); sample of the series H-IS (aged); sample of the series L-IS. For experimental patterns, the experimental data (red points) and the Rietveld refinements (black lines) are reported. Energy: 22 keV, λ : 0.56433Å.

Figure 4 – SEM micrograph of (A) particles of L-IS experiments, scale 500 nm; (B) particles of C-IS experiments, scale 500 nm.

Figure 5 – TEM micrographs of (A,B) L-IS sample and (C,D) C-IS sample.

Figure 6 – Chemical potential plot of literature data [34]. The shaded blue area identify sample with OCP structure whereas the pink shade area identify samples with HA structure.

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Figure 7 – Chemical potential plot of for series H-IS (A), and for series L-IS (B).

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