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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.

Statement of Significance

The formation mechanism of calcium phosphates under biomimetic conditions is unraveled. The formation pathway is mathematically described based on a thermodynamic-kinetic model in which (i) the nucleation stages (primary and secondary) are dominated by the formation of dicalcium phosphate dihydrate (DCPD) and (ii) the fast growth stage is limited by the diffusion of Ca^{2+} ions under the driving force of octacalcium phosphate (OCP), or hydroxyapatite (HA), solubility. The obtained solid phase seems correlated to the activity coefficient of phosphate ions, thus to the ionic strength and local phosphate speciation. The model, being able to highlight the details of the precipitation pathway, is expected to contribute to the understanding of the apatitic phase formation in the biomineralization-biodemineralization processes under *in-vivo* conditions.

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, $Ca_{10}(PO_4)_6X_2$, where X corresponds to OH in hydroxyapatite (HA), and those related to apatite-like structures, such as the octacalcium phosphate, $Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$ (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

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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 [10–12]. 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 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-biodemineralization 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 Na_2HPO_4/NaH_2PO_4 solution. Experimental data were collected at a fixed temperature, initial concentration of phosphate buffer, and pH (T = 37.0 °C ± 0.2 °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 3 solid phases namely dicalcium phosphate dihydrate (DCPD), OCP, and HA. Additional to the system of 14 equations associated with the chemical equations, 2 mass balance equations for total Ca and total P are included. Thus, the problem can be solved if 6 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.