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Amorphous surface layer *versus* transient amorphous precursor phase in bone – A case study investigated by solid-state NMR spectroscopy

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ABSTRACT

The presence of an amorphous surface layer that coats a crystalline core has been proposed for many biominerals, including bone mineral. In parallel, transient amorphous precursor phases have been proposed in various biomineralization processes, including bone biomineralization. Here we propose a methodology to investigate the origin of these amorphous environments taking the bone tissue as a key example. This study relies on the investigation of a bone tissue sample and its comparison with synthetic calcium phosphate samples, including a stoichiometric apatite, an amorphous calcium phosphate sample, and two different biomimetic apatites.

To reveal if the amorphous environments in bone originate from an amorphous surface layer or a transient amorphous precursor phase, a combined solid-state nuclear magnetic resonance (NMR) experiment has been used. The latter consists of a double cross polarization $^{1}H \rightarrow {}^{31}P \rightarrow {}^{1}H$ pulse sequence followed by a ^{1}H magnetization exchange pulse sequence. The presence of an amorphous surface layer has been investigated through the study of the biomimetic apatites; while the presence of a transient amorphous precursor phase in the form of amorphous calcium phosphate particles has been mimicked with the help of a physical mixture of stoichiometric apatite and amorphous calcium phosphate. The NMR results show that the amorphous and the crystalline environments detected in our bone tissue sample belong to the same particle. The presence of an amorphous surface layer that coats the apatitic core of bone apatite particles has been unambiguously confirmed, and it is certain that this amorphous surface layer has strong implication on bone tissue biogenesis and regeneration.

Statement of Significance

Questions still persist on the structural organization of bone and biomimetic apatites. The existing model proposes a core/shell structure, with an amorphous surface layer coating a crystalline bulk. The accuracy of this model is still debated because amorphous calcium phosphate (ACP) environments could also arise from a transient amorphous precursor phase of apatite.
Here, we provide an NMR spectroscopy methodology to reveal the origin of these ACP environments in bone mineral or in biomimetic apatite. The ^{1}H magnetization exchange between protons arising from amorphous and crystalline domains shows unambiguously that an ACP layer coats the apatitic crystalline core of bone et biomimetic apatite platelets.

1. Introduction

The presence of an amorphous surface layer that coats a crystalline nanoparticle core has been proposed both in calcium phosphate and calcium carbonate biominerals, including the apatite nanoparticles constituting the bone mineral [1] and the aragonite particles constituting the nacre of the molluscan shells [2], respectively. In parallel, both transient amorphous calcium phosphate [3,4] and transient amorphous calcium carbonate [5–7] phases have been observed at the early stages of biomineralization in bone, enamel, the spines of sea urchin and in nacre. Here we propose a methodology to investigate the origin of these amorphous environments in the case of a bone tissue sample originating from a two-year-old sheep and through its comparison with synthetic calcium phosphate samples.

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While there is no doubt that the mature bone mineral is made of crystalline calcium phosphate in the form of apatite [8], the question regarding the presence or not of amorphous calcium phosphate (ACP) in bone mineral has been deeply investigated over the past couple of decades [9], and is still widely debated nowadays [10–12]. The bone apatite particles are in the form of nanosized platelets with irregular shapes and dimensions [13,14], *i.e.*, ~1–4 nm of thickness, ~8–15 nm of width and ~20–35 nm of length. This bone apatite is described as a calcium-deficient and hydroxyl-deficient carbonated hydroxyapatite [15–17], with a chemical composition that varies greatly with respect to the so-called stoichiometric hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) due to numerous ionic substitutions – and, in particular, carbonate ions present from ~4 up to ~9% w/w. Moreover, spectroscopic investigations, microscopy observations, and X-ray analyzes, have provided strong evidence of the presence of non-apatitic environments closely related to ACP in bone mineral. Studies involving newly formed, intermediate, and mature bone mineral, as well as synthetic biomimetic apatites (*i.e.*, synthetic carbonated nanocrystalline hydroxyapatites) have been reported – and it was proposed that these ACP environments might have two origins: *(i)* an ACP-like surface layer that coats the bone apatite particles; and/or *(ii)* a transient ACP phase present at the early stages of bone mineralization. Indeed, the existing model that describes the bone apatite and biomimetic apatite particles predict a core/shell structure, with the ACP-like environments in the form of a very thin mineral surface layer that coats the apatitic environments localized into the bulk of the particle. The accuracy of this model is still debated because the exact origin of the ACP environments is still not clear: transient phase *vs* surface layer, either one or the other, or both of the two forms present at the same time in a bone sample.

Particular surface properties of bone mineral were first reported in the fifties from ionic exchange experiments, which would involve "a surface hydration shell containing non-specific boundary anions in rapid equilibrium with the surrounding medium" [18]. The first electron microscopy studies on the early stages of bone mineral deposition suggested that "the crystals are not only smaller, but that some of the observed inorganic components in newly formed bone may not be in a crystalline form" [19]. The presence of ACP as an intermediate in the precipitation of apatite was confirmed *in vitro* in the mid-sixties with the help of X-ray diffraction [20]. Soon after this, based on X-ray diffraction analysis by measuring the background intensity, ACP environments were both detected and quantified in bone mineral [21–23]. The first spectroscopic evidence of the presence of ACP environments in bone mineral was reported at that time using infrared spectroscopy by analyzing the ν_4 vibration mode of orthophosphate ions [24]. However, in the early eighties, with the help of both X-ray diffraction and X-ray radial distribution measurements, M. Glimcher and his colleagues undertook an extensive study that led to the conclusion that the presence of ACP in bone mineral has to be reconsidered [25–27]. Indeed, they concluded that the ACP environments in newly formed bone mineral, previously reported by A. Posner and his colleagues, were actually only "poorly crystalline hydroxyapatite" environments [26]. Other spectroscopic evidences of the presence of ACP or "poorly crystalline hydroxyapatite" environments in bone mineral were reported in 1994 using solid-state nuclear magnetic resonance (NMR) spectroscopy with the help of the $\{^1H\}^{31}P$ cross polarization (CP) experiment [28,29]. These ACP-like environments have been described as "a unique protonated phosphate group in bone mineral not present in synthetic calcium phosphates" [29], as well as "a second phosphorus site interacting with structural water molecules" [28]. In parallel, several studies have reported the presence of related ACP-like environments in various biomimetic apatites, using infrared spectroscopy [30–32], X-ray radial distribution analysis [33], X-ray absorption spectroscopy [34], and solid-state NMR spectroscopy [35–37]. Furthermore, ionic exchange properties involving the presence of loosely bound ions at the particle's surface has also been reported for biomimetic apatites [38–40]. These two concepts involving surface properties and ACP-like environments have therefore gradually merged together, and the models of the bone and biomimetic apatite particles have emerged [1,41–45]: a nanosized platelet constituted by a crystalline core in the form of hydroxyapatite coated by an ACP-like surface layer. Furthermore, a similar core/shell model has also been proposed for the elongated dentin apatite particles [46–48]. High-resolution transmission electron microscopy (HR-TEM) observations have strengthened the accuracy of this model. Indeed, the presence of an amorphous surface layer (~1–2 nm thick) was sometimes reported from synthetic apatite samples [1,49,50]. However, such observations are debatable because a degradation of the particles might happen following the electron beam irradiation.

In parallel, the presence of ACP environments in bone mineral in the form of a transient amorphous precursor phase is worth considering. Indeed, ACP particles have been observed at the early stages of the apatite crystallization process both *in vitro* (with or without the presence of organic additives) [20,51–59] and *in vivo* [3,4,60–62]. In particular, in the case of the growing fin bony rays of the zebrafish, recent studies have shown that the new mineral is delivered and deposited as packages of ACP nano-spheres, which transform into platelets of crystalline apatite within the collagen matrix [63].

The aim of this study is to assess the two scenarios likely to happen in bone mineral: *(1)* an ACP-like surface layer which coats an apatitic crystalline nanoparticle core; and/or *(2)* the presence of ACP particles and apatite crystals apart. For this purpose, a combined solid-state NMR experiment has been applied to a bone tissue sample originating from a two-year-old sheep and to various well-characterized and carefully chosen synthetic calcium phosphate samples. This includes: *(i)* two biomimetic apatites samples composed by the addition of apatitic and ACP-like environments in the same particle; and *(ii)* a physical mixture of a stoichiometric apatite sample (only composed of apatitic environments) and an amorphous calcium phosphate sample (only composed of amorphous environments). The two biomimetic apatites have been used to mimic the scenario *(1)*, while the physical mixture of the stoichiometric apatite and the amorphous calcium phosphate samples has been used to mimic the scenario *(2)*. The solid-state NMR experiment consists of (*i*) a double cross polarization (CP) $^1H \rightarrow {}^{31}P \rightarrow {}^1H$ pulse sequence [64], in order to edit the ^1H resonances related to calcium phosphate environments, followed by *(ii)* a ^1H magnetization exchange pulse sequence based on the ^1H spin diffusion process (EXchange SpectroscopY, EXSY) in order to probe the continuity between the apatitic and the amorphous environments.

2. Materials and methods

2.1. Samples preparation

The stoichiometric hydroxyapatite, **HA**, was obtained following the synthesis described by Takemoto et al. [65]. The double-distilled water used in the different steps of the synthesis has been boiled for 2 h, and then cooled under N_2 bubbling to remove the dissolved CO_2. Briefly, 100 mL of a 0.3 M $(NH_4)_2HPO_4$ aqueous solution (pH fixed at 10 with the help of a 28% w/w ammonia aqueous solution) was added to 105 mL of a 0.5 M $Ca(NO_3)_2{\cdot}4H_2O$ aqueous solution at room temperature under an N_2 atmosphere and rigorous stirring at a feeding rate of 3 mL/min controlled by an auto-