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Protein-free formation of bone-like apatite: New insights into the key role of carbonation

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ABSTRACT

The nanometer-sized plate-like morphology of bone mineral is necessary for proper bone mechanics and physiology. However, mechanisms regulating the morphology of these mineral nanocrystals remain unclear. The dominant hypothesis attributes the size and shape regulation to organic-mineral interactions. Here, we present data supporting the hypothesis that physicochemical effects of carbonate integration within the apatite lattice control the morphology, size, and mechanics of bioapatite mineral crystals. Carbonated apatites synthesized in the absence of organic molecules presented plate-like morphologies and nanoscale crystallite dimensions. Experimentally-determined crystallite size, lattice spacing, solubility and atomic order were modified by carbonate concentration. Molecular dynamics (MD) simulations and density functional theory (DFT) calculations predicted changes in surface energy and elastic moduli with carbonate concentration. Combining these results with a scaling law predicted the experimentally observed scaling of size and energetics with carbonate concentration. The experiments and models describe a clear mechanism by which crystal dimensions are controlled by carbonate substitution. Furthermore, the results demonstrate that carbonate substitution is sufficient to drive the formation of bone-like crystallites. This new understanding points to pathways for biomimetic synthesis of novel, nanostructured biomaterials.

Bone is a composite material composed of the protein type I collagen and plate-like nanocrystals of mineral. The nanometer scale of the mineral crystallites has been shown to be essential to proper bone function and physiology. The nano-size allows the crystallites to be flaw tolerant, thereby optimizing the fracture strength of the bone [1]. In addition, the large surface area of the nano-plates increases the dissolution rate, which allows the body to quickly release necessary ions in response to pathologies such as acidosis [2–4]. Although the nanocrystal size and morphology are essential to bone's mechanical and physiological functions and must be considered in the creation of bone replacement biomaterials, the mechanism by which these properties are controlled remains unclear.

The mineral phase in bone is often described as hydroxylapatite, a natural geologically occurring OH^--containing calcium apatite. Geological hydroxylapatite forms large hexagonal prisms that derive from its hexagonal lattice structure [5]. However, the mineral in bone resembles nanometer-scale elongated plates or blades while still maintaining an internal hexagonal lattice structure

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[6–12]. More precisely, the nanocrystals of both bone and biomimetic apatites exhibit a complex substructure composed of (1) an apatitic, often nonstoichiometric core containing ions in apatitic (i.e., lattice) chemical environments and (2) a surface layer containing ions in non-apatitic (i.e., non-lattice) environments and surrounded by water molecules [13]. In order to explain this discrepancy between the morphology of geological (inorganic) and biological apatites, many researchers have suggested that bone's crystallite size and shape are controlled *via* templating by or chemical interactions with surrounding proteins and organic moieties [10–12,14,15]. It has been theorized that the fibrillar structure of collagen dictates the size of the crystallites *via* physical confinement within its nanometer-sized gap zones [12,16]. Others propose that proteins or other organic moieties bind to the mineral surface and control crystal growth [10,11,14,15,17]. These results seem to suggest that organic interactions are necessary for the formation of plate-like nanocrystals. However, what many of these studies fail to consider is the true composition of bioapatite.

The apatite structure is remarkably accommodating to substitutions [18]. The most common substitutions seen in bone mineral are the coupled substitution of CO_3^{2-} for PO_4^{3-} and Na^+ for Ca^{2+}, which lead to vacancies on the calcium and hydroxyl sites [18–20]. As a result, bone mineral is more accurately described as a carbonated, calcium- and hydroxyl-depleted apatite. The substitution of carbonate into the apatite structure can occur at one or both of two sites: in exchange for hydroxyl (OH^-), defined as A-type, or for phosphate (PO_4^{3-}), defined as B-type [21]. In mammalian bone, carbonate levels span 2–8 wt%, depending on species and bone location, and are predominantly of B-type substitution [18,22–24]. Empirical observations have qualitatively shown that carbonate content in the apatite lattice affects crystallite size [25–29], solubility [8,30,31], and crystal mechanics [32]. These results suggest that, in addition to possible organic interactions, carbonate concentration plays an essential role in regulating bone mineral morphology. Despite the importance of carbonate substitution to crystal properties, there is no proposed mechanism for this process. We hypothesized that, in the absence of proteins or other organic substances, carbonate substitution, through its control of crystal lattice spacing, atomic order, and solubility, is sufficient to control apatite crystals' nano-scale size, shape, and mechanics *via* energetic effects [8,26,30,31]. This work proposes, for the first time, a direct mechanism for substitutional effects on crystallite morphology.

A protein-free solution precipitation technique was used to prepare biomimetic apatites with carbonate concentrations ranging from 1.0 to 17.8 wt%. The composition, gross morphology, and lattice structure were measured experimentally, providing a comprehensive analysis of biomimetic carbonated apatites. Molecular dynamics (MD) and density functional theory (DFT) calculations, which were validated through comparison to experimental results, evaluated the energetics (i.e., conservation of energy and a trade-off between surface energy and strain energy) of carbonate substitution. This work presents a situation in which nanoparticles are not created *via* templating to enhance the chemical properties, but rather in which the chemistry itself imposes a nanoscopic size. Organic templating is not necessary to form crystallites that exhibit physiological structures; rather, control of carbonate substitution is sufficient to precipitate crystallites with the correct nanometer-scale size and morphology. Experimental and theoretical parameters from these analyses were used in a continuum analysis to derive a proposed mechanism for how carbonate substitution controls crystallite size. Understanding this relationship provides new insight into the formation of biomimetic crystals for tissue engineering.

1. Methods

1.1. Synthesis of powders

Apatite powders were made according to previously described methods [33–35] and summarized here. All water used in the synthesis was either deionized water that was further purified by passing through a calcium hydroxylapatite column or doubly deionized Milli-Q (18 MΩ) water in order to minimize the amount of fluoride present. Nitrogen was then bubbled through the water for several hours in order to remove dissolved carbon dioxide. Approximately 250 mL of water was placed in a large beaker and heated to the desired reaction temperature (usually 60 °C or 85 °C). Mixtures were stirred magnetically. In accordance with the desired degree of carbonate substitution, an appropriate amount of $NaHCO_3$ or Na_2CO_3 (Acros-Organics, 99.5% or GFS Chemicals, Primary Standard) was dissolved directly in the 250 mL reactor bath, and the pH was elevated to 9.0 ± 0.5 and maintained with 0.1 M NaOH (Acros, 97%). To the bath, 25 mL of a 0.15 M $Ca(NO_3)_2{\cdot}4H_2O$ (Sigma-Aldrich, 99%) and 25 mL of a 0.09 M NaH_2PO_4 (Alrich, 98%) solution were added at a rate of 1 drop/2–4 s. After addition was complete, the mixture was digested at temperature for 2 h. After the digestion, heating and stirring were discontinued. The mixture was cooled to room temperature and then filtered in a medium porosity glass filter crucible. The product was washed three times with warm water, air dried in a desiccator overnight, and then dried in a 120 °C vacuum pistol or an evacuated vacuum desiccator for a minimum of 24 h. 20 powders were made with CO_3^{2-}/PO_4^{3-} molar ratios ranging from 0.2 to 12 (Table 1).

1.2. Carbonate concentration determination

1.2.1. CHN analysis

In order to accurately measure the carbonate concentration of the apatite powders Carbon-Hydrogen-Nitrogen (CHN) analysis was performed by Galbraith Labs, Knoxville, TN. Analysis was performed by combusting the samples and measuring the levels of CO_2, H_2O and N_2 (or NO_x) by either an infrared cell or a thermal conductivity detector. Levels were reported as weight percents (wt %).

1.2.2. Raman analysis

Raman analysis was also used to determine the carbonate concentration as well as analyze the atomic order. The Raman microprobe apparatus (HoloLab Series 5000 fiber optically coupled Raman Microscope, Kaiser Optical Systems, Inc) has been previously described [36]. Spectra were acquired using a 532 nm laser focused to a ~1 μm spot size by an 80× objective (N.A. = 0.85) at a power of 10 mW on the sample surface. Scattered light was collected in 32 4-sec acquisitions through the objective lens and transferred to a 2048-channel CCD detector. Powders were manually compressed before analysis, and spectra were obtained at six independent locations within each powder.

Spectra for 20 of the powders were analyzed as described previously [36–39]. Briefly, the spectra were background corrected in the region of 700–1200 Δcm^{-1} and the peaks were deconvolved into their component bands by applying a mixed Gaussian-Lorentzian peak fitting algorithm using the Grams32 software package (Galactic, Salem, NH). The relative carbonate concentration was determined from the ratio of the areas for the band associated with carbonate substituting for phosphate, i.e. 1070 Δcm^{-1}, and the ν_1 P-O stretch band of hydroxylapatite at 960 Δcm^{-1} (Fig. 1A). Weight percent of carbonate was determined from a calibration curve developed in our laboratory. In addition, full width at half maximum (FWHM) of the 960 Δcm^{-1} peak was used to evaluate