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Crystallinity and compositional changes in carbonated apatites: Evidence from ^{31}P solid-state NMR, Raman, and AFM analysis

John-David P. McElderry^a, Peizhi Zhu^a, Kamal H. Mroue^{a,b}, Jiadi Xu^b, Barbara Pavan^d, Ming Fang^a, Guisheng Zhao^c, Erin McNerny^c, David H. Kohn^c, Renny T. Franceschi^c, Mark M. Banaszak Holl^a, Mary M.J. Tecklenburg^{d,*}, Ayyalusamy Ramamoorthy^{a,b}, Michael D. Morris^a

^a Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA

^b Department of Biophysics, University of Michigan, Ann Arbor, MI 48109-1055, USA

^c School of Dentistry, University of Michigan, Ann Arbor, MI 48109-1055, USA

^d Department of Chemistry and Science of Advanced Materials Program, Central Michigan University, Mt. Pleasant, MI 48859, USA

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ABSTRACT

Solid-state (magic-angle spinning) NMR spectroscopy is a useful tool for obtaining structural information on bone organic and mineral components and synthetic model minerals at the atomic-level. Raman and ^{31}P NMR spectral parameters were investigated in a series of synthetic B-type carbonated apatites (CAps). Inverse ^{31}P NMR linewidth and inverse Raman PO_4^{3-} ν_1 bandwidth were both correlated with powder XRD *c*-axis crystallinity over the 0.3–10.3 wt% CO_3^{2-} range investigated. Comparison with bone powder crystallinities showed agreement with values predicted by NMR and Raman calibration curves. Carbonate content was divided into two domains by the ^{31}P NMR chemical shift frequency and the Raman phosphate ν_1 band position. These parameters remain stable except for an abrupt transition at 6.5 wt% carbonate, a composition which corresponds to an average of one carbonate per unit cell. This near-binary distribution of spectroscopic properties was also found in AFM-measured particle sizes and Ca/P molar ratios by elemental analysis. We propose that this transition differentiates between two charge-balancing ion-loss mechanisms as measured by Ca/P ratios. These results define a criterion for spectroscopic characterization of B-type carbonate substitution in apatitic minerals.

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1. Introduction

Crystallinity is generally regarded as one of several metrics that are used to define optimum bone material properties. Shifting of bone mean crystallinity and increasing crystallinity variability over time have been associated with bone fragility [1,2]. Bone mineral crystallinity generally increases with age [3,4]; and higher crystallinity is characteristic of osteoporotic bone [5–7].

Bone is a dual-phase material composed of an organic matrix reinforced with a rigid mineral. The main component of the organic phase is type I collagen (~90%), along with small amounts of heterotypic collagens and various non-collagenous proteins and polysaccharides [8,9]. Bone mineral, on the other hand, has a structure similar to the mineral calcium hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. However, unlike calcium hydroxyapatite, bone mineral is poorly crystalline and nonstoichiometric. In addition to very small amounts of other ions

(such as Na^+ , Mg^{2+} , F^-), bone mineral contains 5–8 wt% CO_3^{2-} . Predominantly, the carbonate is present as B-type carbonate, i.e. with carbonate substituting for phosphate ions [10,11].

Synthetic B-type carbonated apatites (CAps) are commonly used as model compounds for investigating chemical properties of bone mineral, and have been the subject of extensive research due to their importance in a variety of biomedical applications such as grafts and scaffolds for reconstructing or regenerating bone defects, osteoconductive implant coatings, and vehicles for drug delivery [12,13]. Several research groups have synthesized bone-like apatites under near-physiological conditions [14,15]. The biological response to synthetic apatites, both in vitro and in vivo, depends, in part, on the degree of carbonation and crystallinity of the apatite.

In X-ray diffraction (XRD) crystallography, apatite crystallinity is conventionally defined by the Debye–Scherrer equation, where average size of the crystalline domains along a crystallographic dimension is inversely proportional to the full-width at half-maximum (FWHM) of a reflection band along that axis. *c*-axis crystallinities were used for apatite sizing throughout this study

* Corresponding author. Fax: +1 989 774 3883.

E-mail address: mary.tecklenburg@cmich.edu (M.M.J. Tecklenburg).

because crystallites in bone are elongated mostly along the *c*-axis [16,17]. Lattice strain also contributes to reflection band broadening, which can have a contribution in imperfect crystallites. Although crystal length measures can be separated from lattice strain as part of a Rietveld refinement, such analyses can introduce a considerable amount of error and are not often used in studies of nonstoichiometric minerals and bone, where crystallinity is typically low [3,18,19].

Similar crystallinity/inverse bandwidth relationships are also used in spectroscopic techniques for mineral analysis. Fourier transform infrared spectroscopy (FTIR) has long been used for measuring mineral crystallinity in bone and apatite [7,20]. While FTIR bandwidths are sensitive to crystallinity, band area ratios also indicate crystallinity and are the preferred metric for most bone applications [7,20–22]. Akkus and co-workers have correlated CAP crystallinity and inverse Raman bandwidths, and have applied these correlations to bone mineral [23–25]. Similar Raman/crystallinity correlations have been reported by Kazanci et al. [26] and Turunen et al. [27]. Linear correlations with powder diffraction data rather than calculations from a governing equation are usually used to define crystallinity in these techniques.

Solid-state NMR spectroscopy is a powerful tool to obtain high-resolution structural information on apatite and bone mineral at the atomic-level [28–33]. ^{31}P and ^{43}Ca magic-angle spinning (MAS) NMR experiments have been used to study structural changes in apatite and bone mineral [34–40]. Over the past decade, solid-state NMR methodologies have also been used to study the crystallinity of semi-crystalline polymers [41–43]. To the best of our knowledge, no analogous correlations have been reported for solid-state nuclear magnetic resonance (NMR) data on CAPs or bone, although broader NMR line shapes have been observed in disordered minerals [38,44,45].

In this study, we hypothesized that apatite crystallinity measures are obtainable from ^{31}P NMR spectra and that an inverse linewidth relationship exists, similar to Raman spectra of bone mineral. We undertook a systematic study, by NMR and Raman spectroscopy, of the crystallinity of synthetic carbonated apatites and we investigated the applicability of the same measures to two examples of bone mineral from bovine femur and murine femur. The bone samples are representative of the typical carbonate levels in bone mineral while the synthetic CAPs span a much broader range of carbonate substitution than naturally occurs in bone. The results from ^{31}P MAS NMR measurements on the series of B-type CAPs with various carbonate levels were analyzed and compared with crystallinity measurements performed by powder X-ray diffraction (XRD) and Raman spectroscopy. ^{31}P NMR isotropic chemical shifts and their linewidths (FWHM) were extracted from the experimentally measured line shapes, and related to percent carbonation. Bovine and murine bone powders were also studied by these spectroscopies and compared to the CAPs. While crystallinity by XRD provides information about the crystal domain size, we supplemented our measurements with atomic force microscopy (AFM) as a direct measure of total particle size irrespective of axis orientation. AFM has been used as a direct measure of bone mineral nanoparticles, and more recently, periodicity of bone matrix spacings [18,46,47].

In addition, we explored the nature of carbonate composition dependence on apatite crystallinity. We observed that NMR and Raman spectroscopy show similar carbonate composition-dependent variation in the position of ^{31}P isotropic chemical shift and the wavenumber of the $\text{PO}_4^{3-} \nu_1$ symmetric stretch. The composition dependence is nearly binary and remarkably consistent across analysis techniques; however, this dependence was not observed in the crystallinity metrics associated with NMR linewidths and Raman bandwidths. We propose that different mechanisms of charge balancing ion-loss (ion vacancies) account for the differences in spectral frequency between low and high levels of carbonate substitution in

apatitic minerals. We anticipate that our results will contribute to an improved understanding of ion substitution in synthetic and natural apatitic minerals and will broaden the capabilities of solid state NMR in that pursuit.

2. Materials and methods

2.1. Sample preparation

Our study includes a series of synthetic carbonated apatites (CAPs) in addition to bovine femoral bone (BF) powder taken from the mid-diaphyseal cortex and powder from six-month old murine whole femora (MF). The CAP series was synthesized at low temperatures (80–90 °C) following the aqueous precipitation method published by Penel et al. for B-type apatites [48]. Variations in the published method were limited to replacing sodium phosphate and sodium carbonate with the corresponding ammonium salts for the purpose of reducing cationic substitution into Ca(I) and Ca(II) sites.

Composition of the synthetic CAP samples was determined by ICP-OES for calcium and phosphorus content using a Perkin Elmer Optima 3000. Emission lines used were 317.9333 nm for Ca and 213.617 nm for P (under N_2 purge). The samples were dissolved in 2% wt/vol nitric acid in ultrapure water (resistivity 17–18 Mohm cm, < 0.03 ppm dissolved solids). Standard calcium and phosphorus solutions from Perkin Elmer were used for calibration and a standard hydroxyapatite sample from the National Institute for Standards and Technology (NIST; SRM 2910) was used as a check standard.

FTIR spectra for carbonate analysis were acquired using a Nicolet Magma FTIR spectrometer (Thermo Electron Corp., Madison, WI) with a KBr beamsplitter and DTGS detector on KBr pellets. At a resolution of 4 cm^{-1} , 250 scans were ratioed against an air background and the spectra were processed to obtain baselines and peak heights in absorbance units using Omnic software (Thermo Electron Corp.).

The carbonate content of ten apatite samples, with carbonate levels that varied from 0.2 to 10.3 wt%, was determined by coulometric titration after heating and collecting CO_2 gas in an absorption cell containing lithium hydroxide (Galbraith Laboratories, Knoxville, TN). A calibration curve based on the absorbance ratio of FTIR peaks at 1454 cm^{-1} (carbonate) to 565 cm^{-1} (phosphate) versus wt% carbonate (by chemical analysis) was used to determine the carbonate content of the CAP samples used in this study.

Bovine femora were harvested from freshly slaughtered animals (2–4 years old). Femora were stripped of soft tissue, and cortical bone specimens were prepared from central diaphyseal sections. Each diaphysis was sectioned on a band saw into parallelepipeds. Calcium-buffered saline was used during all machining steps to avoid heating the bone and to maintain tissue saturation and ionic balance. Sections randomly chosen from an inventory of ten femora with respect to longitudinal and circumferential location were milled into a powder while cryogenically cooled with liquid nitrogen. Right and left femora were collected from two mice, (male, C57BL/6, age 6 months), and milled into a powder in a similar fashion to the bovine bone powder. Carbonate content for these biological specimens was determined by comparing the Raman carbonate-to-phosphate ratio of the 1070 cm^{-1} (carbonate) and $\sim 960\text{ cm}^{-1}$ (phosphate) bands, as described in Ref. [49], to a calibration curve consisting of carbonate-to-phosphate values measured from the CAP series.

2.2. Nuclear magnetic resonance experiments

All solid-state ^{31}P NMR experiments were carried out on a Varian/Agilent VNMRJ 600 MHz NMR spectrometer (operating at a Larmor frequency of 242.8 MHz for ^{31}P) equipped with a 4-mm

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