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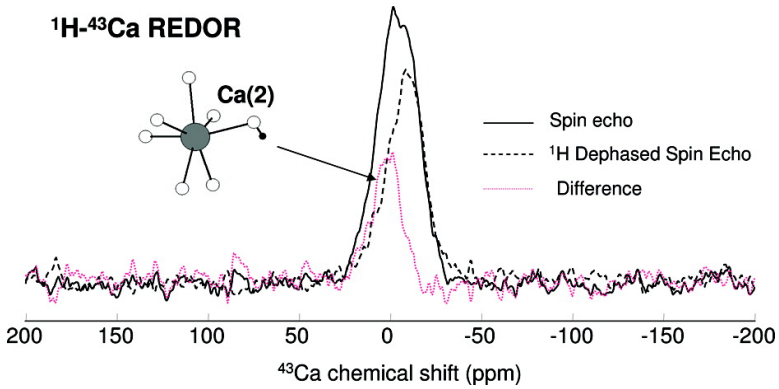

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A High-Resolution ^{43}Ca Solid-State NMR Study of the Calcium Sites of Hydroxyapatite

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Calcium is the fifth most abundant element in the Earth's crust, where it can mainly be found associated with carbonates and sulfates, in minerals such as calcite, aragonite, and gypsum.[1] Furthermore, calcium is one of the most common metals in many living organisms.[2] It is the major cation in biominerals like hydroxyapatite, and binds to proteins both in inner- and extracellular environments. The broad diversity of structural, physiological, and biochemical roles played by calcium compounds undoubtedly relies on the ability of Ca^{2+} cations to take a large variety of coordination environments. Unfortunately, very few spectroscopic techniques are available to probe the local structure around calcium atoms, thus hampering a thorough understanding of a large number of biological processes. Although single-crystal X-ray diffraction (XRD) has in some cases provided information on the coordination sphere of calcium,[2,3] this technique unfortunately cannot be used for the study of many calcium species because they do not form crystals suitable for XRD or because they are available only in amorphous or glassy forms. Other experimentally challenging techniques have thus been used, such as Ca K-edge X-ray absorption spectroscopy[4−9] and ^{43}Ca solid-state NMR.

^{43}Ca is a spin $^7/_2$ isotope with a small magnetic moment (termed low-γ) of very low natural abundance (0.14%), making it a difficult nucleus for NMR.[10] However, thanks to recent instrumental advances (access to high magnetic fields, large volume magic-angle spinning (MAS) probes) and new pulse sequences specific to quadrupolar nuclei, ^{43}Ca solid-state NMR has become more accessible. The few ^{43}Ca studies performed so far in the solid state have shown the potential of this technique in providing valuable structural information;[10−14] in particular, the resolution of different calcium sites by MAS NMR has in some cases been achieved.[11c,12] However, the assignment of resolved calcium signals has so far relied on the knowledge of the single-crystal XRD structure (number of calcium atoms in each site and average Ca···O distances). Since many calcium compounds do not readily crystallize, especially those of biological significance, it thus appears crucial to find other means for differentiating ^{43}Ca NMR signals. Given the variety of ligands which can bind to Ca^{2+} in biological complexes, calcium sites often also differ in the number of protons in their direct vicinity. It would thus be very valuable to be able to distinguish calcium cations according to their respective proton environment. In this work, a hydroxyapatite sample is used to demonstrate that solid-state NMR offers this opportunity by taking advantage of differences in ^{1}H-^{43}Ca dipolar couplings.

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is the main inorganic phase of bone tissue and teeth. In the crystal structure, there are two calcium sites, referred to as Ca(1) and Ca(2) (see Figure 1). The ratio between the Ca(2) and Ca(1) sites is $^3/_2$. The Ca(1) site is coordinated to phosphate oxygens only, whereas the Ca(2) site is linked to both phosphate oxygens and a hydroxyl group.[15]

Figure 1. Illustration of the two calcium coordination environments in hydroxyapatite.[15] (Ca, O, and H atoms are in gray, white, and black, respectively).

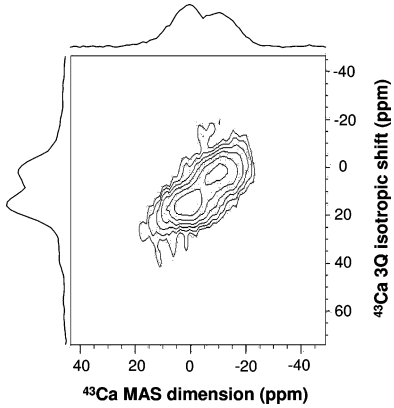

Figure 2. ^{43}Ca 3QMAS spectrum acquired on a Bruker Advance II 600 (14.1 T) spectrometer operating at 40.386 MHz, using a Bruker 4 mm MAS probe and spinning at 8 kHz. Excitation and conversion pulses were set to 8.5 μs and 2.6 μs, respectively. A total of 4800 transients with a 0.05 s recycle delay were recorded for each of 110 t_1 points. Chemical shifts are reported with respect to a saturated solution of $CaCl_2$ at 0 ppm.

Recently, a natural abundance ^{43}Ca MAS solid-state NMR study was performed on hydroxyapatite at three magnetic fields (8.45, 14.1, and 18.8 T). The two calcium sites were resolved at 18.8 T (but not at lower fields), and the NMR interaction parameters of each site (δ_{iso}, C_Q, and η_Q) were determined through multiple magnetic field simulations.[12] The relative intensity of the peaks allowed the assignment of the high ($\delta_{iso} = 11.2 \pm 0.8$ ppm) and low ($\delta_{iso} = -2.6 \pm 0.8$ ppm) frequency signals to the Ca(2) and Ca(1) sites, respectively.

Since more than 24 h were necessary to acquire each ^{43}Ca MAS spectrum at natural abundance, testing more sophisticated NMR sequences likely to differentiate calcium sites on the basis of their environment would have been very time-consuming. A ^{43}Ca-labeled hydroxyapatite was thus synthesized, starting from enriched (60%) $CaCO_3$.[16] The tremendous gain in signal-to-noise is well illustrated in Figure 2: it allowed recording of a 2D triple quantum magic-angle spinning (3QMAS) spectrum at 14.1 T in only 10 h. In contrast to MAS at this field (see Supporting Information), the two peaks are unequivocally resolved in the 3Q dimension, with the 3Q shift positions being in good agreement with the previously determined NMR parameters.[12] Although ^{43}Ca

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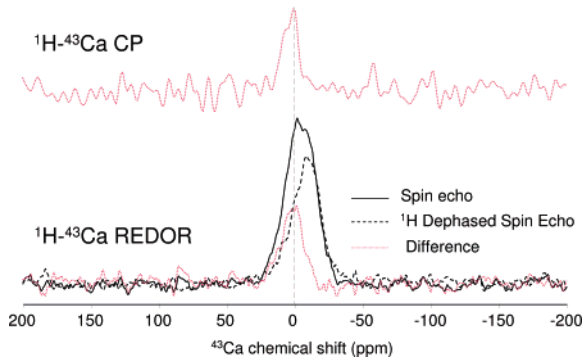

Figure 3. (Top) ^{1}H-^{43}Ca CPMAS NMR spectrum of hydroxyapatite at 14.1 T, spinning at 2.5 kHz, and using a contact time of 1 ms (18600 transients, ~5 h). (Bottom) Non-dephased and dephased ^{1}H-^{43}Ca REDOR spectra at 14.1 T, spinning at 8 kHz (24000 transients, ~1 h per spectrum); $\pi/2$ and π pulses of 1.5 and 3.0 μs respectively were applied to the ^{43}Ca channel. In the dephased spectrum, 29 ^{1}H π pulses were applied on each side of the π pulse (total dephasing time of 2.75 ms).

multiple-quantum MAS NMR spectra of ^{43}Ca-labeled glassy slags have been reported recently,[13] it should be noted that this spectrum is the very first one to unambiguously resolve two calcium sites in a material using 3QMAS.

As shown in Figure 1, in the hydroxyapatite structure, only the Ca(2) site is close to protons, with an average Ca(2)···H distance of ~2.67 Å.[15] The ^{1}H-^{43}Ca(2) dipolar coupling is weak (<425 Hz) since ^{43}Ca is a low-γ nucleus, and it is easily averaged by MAS. Given that dipolar couplings give highly valuable structural information, because of their direct dependence ($\propto r^{-3}$) to the internuclear distance r, specific techniques have been developed to probe dipolar interactions under MAS. In particular, the cross-polarization (CP) and rotational echo double resonance (REDOR)[17] sequences are now routinely used to look at dipolar couplings between spin-$^{1}/_{2}$ nuclei. Though these techniques are much more challenging when it comes to investigating dipolar couplings between spin-$^{1}/_{2}$ and quadrupolar nuclei, they have been successfully applied for structural studies in a few cases.[10,18,19] However, the only ^{1}H-^{43}Ca CPMAS experiment attempted so far led to the destruction of both the probe and sample, because of the very long contact times (up to 70 ms) used.[20]

^{1}H-^{43}Ca REDOR and CPMAS of the labeled hydroxyapatite sample were here performed to see whether the differences in ^{1}H-^{43}Ca dipolar coupling between the two calcium sites could be used to distinguish them. The spectra are shown in Figure 3. On the one hand, comparison of the conventional spin−echo and ^{1}H dephased spin echo spectra in the REDOR experiment shows that there is a striking decrease in intensity of the high-frequency component of the signal in the dephased spectrum. On the other hand, on the CPMAS spectrum, only the high-frequency component of the MAS signal appears. Both of these observations equally show that the high-frequency component of the MAS signal of hydroxyapatite corresponds to calcium cations close to protons. This is in complete agreement with our previous assignment of the high-frequency signal to the Ca(2) site and clearly demonstrates that REDOR and CPMAS experiments are perfectly suited to differentiate calcium sites on the basis of their respective proton environments.

The experiments presented here on hydroxyapatite underscore more generally the high potential of ^{43}Ca solid-state NMR. On the one hand, 3QMAS experiments at 14.1 T can efficiently average out the second-order quadrupolar broadening and lead to high spectral resolution. On the other hand, experiments such as REDOR or CPMAS can be used for assigning calcium coordination environments, thus opening up new perspectives for the analysis of complexes in which calcium cations display differential proton proximities, which is often the case in biological environments. Though the studies were optimized on a labeled sample, it should be noted that we have also obtained first evidence that REDOR could also be done at natural abundance (see Supporting Information). This should greatly encourage the use of ^{43}Ca solid-state NMR as a sensitive, almost unique, spectroscopic tool to probe calcium environments. Given the natural relevance of calcium, fields like biology and the Earth sciences would greatly benefit from the possibility of probing precisely the coordination environment of Ca^{2+} cations by NMR. Furthermore, this work offers new perspectives for the study and understanding of processes like biomineralization and bone formation, in which calcium plays a pivotal role by interacting with both organic and mineral phases.

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Supporting Information Available: ^{31}P, ^{1}H, and ^{43}Ca MAS NMR spectra of the labeled hydroxyapatite sample at 14.1 T. Natural abundance ^{1}H-^{43}Ca REDOR analysis of hydroxyapatite at 14.1 T. This material is available free of charge via the Internet at http://pubs.acs.org.

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