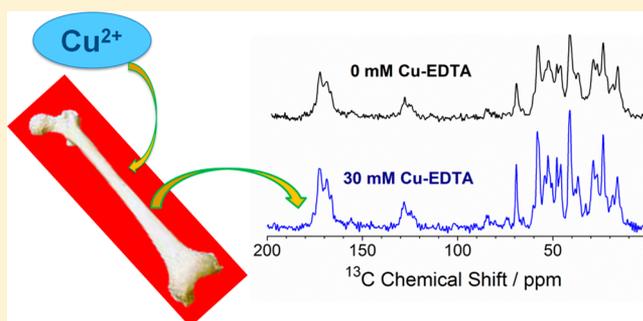


High-Resolution Structural Insights into Bone: A Solid-State NMR Relaxation Study Utilizing Paramagnetic Doping

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ABSTRACT: The hierarchical heterogeneous architecture of bone imposes significant challenges to structural and dynamic studies conducted by traditional biophysical techniques. High-resolution solid-state nuclear magnetic resonance (SSNMR) spectroscopy is capable of providing detailed atomic-level structural insights into such traditionally challenging materials. However, the relatively long data-collection time necessary to achieve a reliable signal-to-noise ratio (S/N) remains a major limitation for the widespread application of SSNMR on bone and related biomaterials. In this study, we attempt to overcome this limitation by employing the paramagnetic relaxation properties of copper(II) ions to shorten the ¹H intrinsic spin-lattice (T_1) relaxation times measured in natural-abundance ¹³C cross-polarization (CP) magic-angle-spinning (MAS) NMR experiments on bone tissues for the purpose of accelerating the data acquisition time in SSNMR. To this end, high-resolution solid-state ¹³C CPMAS experiments were conducted on type I collagen (bovine tendon), bovine cortical bone, and demineralized bovine cortical bone, each in powdered form, to measure the ¹H T_1 values in the absence and in the presence of 30 mM Cu(II)(NH₄)₂EDTA. Our results show that the ¹H T_1 values were successfully reduced by a factor of 2.2, 2.9, and 3.2 for bovine cortical bone, type I collagen, and demineralized bone, respectively, without reducing the spectral resolution and thus enabling faster data acquisition. In addition, paramagnetic quenching of particular ¹³C NMR resonances on exposure to Cu²⁺ ions in the absence of mineral was also observed, potentially suggesting the relative proximity of three main amino acids in the protein backbone (glycine, proline, and alanine) to the bone mineral surface.



INTRODUCTION

The complex hierarchical structure of bone serves to provide the strength, toughness, and stiffness required of the material.^{1–10} Atomic-level structure and dynamics are necessary for understanding the intimate molecular interactions between the organic matrix (primarily type I collagen) and the mineral surface (primarily poorly crystalline calcium-rich carbonated hydroxyapatite) in bone tissues that provide strength and toughness with a minimal amount of mass. Understanding molecular interactions is also a prerequisite for the development of therapeutic approaches to treat bone diseases and regenerate bone defects. However, this is a challenging task for traditionally used characterization techniques^{11–18} due to the heterogeneous and amorphous nature of bone tissues. Recent studies have demonstrated that high-resolution solid-state nuclear magnetic resonance (SSNMR) spectroscopy represents an invaluable method for the characterization of bone tissues and related biological materials, as it has a much higher potential to elucidate structure and dynamics in a quantitative manner on the molecular level. These NMR studies range from one- (e.g., ¹H, ¹³C, ³¹P, and ⁴³Ca) to two-dimensional (e.g., ³¹P/¹H and ¹³C/¹H heteronuclear correlation) magic-angle spinning (MAS) measurements^{19–43} and have markedly

enriched our knowledge and perception about the structure and function of bone.

Notwithstanding the recent developments in NMR instrumentation and methodology, one of the key challenges that still hinders the widespread application of SSNMR spectroscopy for bone and related biomaterials is the long data-collection time required to achieve a reasonable signal-to-noise ratio (S/N) of the NMR spectrum. It is well-known that acquiring an NMR spectrum with excellent sensitivity is crucial for the accurate analysis and interpretation of the NMR line shapes, especially for macromolecules with a large number of amino acid residues. In ¹³C CP MAS experiments, for example, the interscan recycle delay depends exclusively on the proton spin-lattice (T_1) relaxation times and consumes more than 90% of the measurement time in the solid state. Therefore, the development of new approaches and methodologies to shorten these recycle delays and thereby accelerate SSNMR measurements is timely and essential.

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Doping with paramagnetic reagents such as Cu^{2+} can effectively enhance the longitudinal ^1H relaxation rates and accelerate data acquisition in solution as well as in SSNMR on peptides, proteins, and model membranes.^{44–57} Although the previous SSNMR studies have successfully investigated the use of paramagnetic doping to speed up the longitudinal relaxation process in MAS NMR measurements of crystalline peptides/proteins and hydrated lipid bilayers, the effects of paramagnetic dopants on spin–lattice (T_1) relaxation times in heterogeneous and amorphous biomolecular systems have not been extensively explored, particularly for bone. Therefore, in an effort to acquire further understanding of the organic–mineral interface in bone, we present here our high-field SSNMR investigation of the utility of paramagnetic ion doping to enhance ^1H longitudinal relaxation rates in one-dimensional ^{13}C CP MAS for powdered samples of three bone specimens: type I collagen (bovine tendon), fresh bovine cortical bone, and demineralized bovine cortical bone. To this end, we have employed the paramagnetic properties of Cu^{2+} ions using the $\text{Cu}(\text{II})\text{-(NH}_4)_2\text{EDTA}$ complex (henceforth referred to as Cu–EDTA) as a relaxation enhancement agent. Additionally, the application of one of the highest available magnetic fields (21.14 T/900 MHz ^1H Larmor frequency) has enabled us to detect the various ^{13}C NMR resonances in native bone components with high sensitivity at natural abundance, which in turn has facilitated the investigation of the effect of Cu–EDTA doping on both the line widths and line positions of the ^{13}C resonances in the CP MAS spectra for these samples.

EXPERIMENTAL SECTION

Sample Preparation. Bovine femora were collected from freshly slaughtered animals (2–4 years old) at a local abattoir. After cleaning of all soft tissues, slices of cortical bone were dissected from mid-diaphyseal femoral regions and machined into rectangular specimens with a band saw under continuous irrigation with calcium-buffered saline solution to avoid excessive heating of the bone pieces. Rectangular specimens, randomly selected from a collection of 10 femora with respect to longitudinal and transverse orientation, were ground into powder in a cryogenic mill under liquid nitrogen. Type I collagen (bovine tendon) was purchased from Sigma Aldrich (St. Louis, MO). For consistency, a sample of type I collagen was also milled into fine powder while cryogenically cooled with liquid nitrogen. Finally, the obtained powdered samples were soaked with standard phosphate-buffered saline (PBS) solution and stored at $-20\text{ }^\circ\text{C}$ prior to further treatment and NMR measurements.

Powdered bovine bone was demineralized in 0.2 N hydrochloric acid solution for 5 h at room temperature,⁵⁸ then washed in excess of PBS solution, and filtered. A 30 mM Cu–EDTA PBS solution was prepared by dissolving $\text{Cu}(\text{II})\text{-(NH}_4)_2\text{EDTA}$ powder (Sigma Aldrich, St. Louis, MO) in standard PBS buffer. Bone and collagen samples were suspended and mixed with a 30 mM Cu–EDTA PBS solution for about 10 min and filtered for each NMR experiment.

NMR Spectroscopy. Preliminary NMR experiments were performed on a Varian VNMRJ 600 MHz solid-state NMR spectrometer using a 4 mm double-resonance MAS probe. To acquire high-resolution ^{13}C NMR spectra with better sensitivity, all solid-state NMR experiments reported herein were performed at 21.14 T on a Bruker AVANCE 900 MHz spectrometer operating at a resonance frequency of 226.3 MHz for ^{13}C and 899.9 MHz for ^1H , and equipped with a 4-mm

triple-resonance magic angle spinning (MAS) probe. The ^{13}C MAS spectra were acquired under 10 kHz MAS conditions at $25\text{ }^\circ\text{C}$ using a ramped-amplitude cross-polarization (Ramp-CP) pulse sequence during an acquisition period of 5 ms with a 2 ms cross-polarization contact time. A 75 kHz two-pulse phase-modulation (TPPM)⁵⁹ scheme was applied to decouple protons during signal acquisition. ^{13}C NMR chemical shifts were referenced with respect to TMS using adamantane as a secondary external standard. The ^1H T_1 values were calculated from a series of data collected by ^1H spin-inversion recovery experiments detected in ^{13}C Ramp-CP MAS, in which a ^1H π -pulse followed by an inversion recovery delay were introduced prior to the Ramp-CP pulse sequence and TPPM decoupling scheme. Six data points with increasing inversion recovery delays were recorded for each sample. The recycle delays were set to 7 s for samples containing no Cu–EDTA and 3 s for those with 30 mM Cu–EDTA, and 2000 scans were accumulated for each inversion recovery experiment. The signal intensities were measured for peaks in the aliphatic (10–70 ppm) and the carboxyl/carbonyl (165–185 ppm) regions to determine the corresponding ^1H T_1 values. Only those residues whose signals are distinctly resolved in the ^{13}C MAS spectrum and have adequate S/N have been considered for data analysis. The average of the ^1H T_1 values determined for these residues was adopted as the ^1H T_1 value of the sample in question.

RESULTS AND DISCUSSION

Prior to commencing the discussion on the SSNMR results, a comment should be made on the bone demineralization procedure used in this study. A common bone demineralization procedure is treatment with EDTA.⁶⁰ Therefore, it is reasonable to question whether the use of $\text{Cu}(\text{II})\text{-(NH}_4)_2\text{EDTA}$ as the paramagnetic relaxation agent would cause the exchange of Cu^{2+} for Ca^{2+} ions in the hydrated surface layer of bone or within the inorganic crystal lattice. For comparison, the log formation constant for the copper–EDTA complex is 18.8, whereas the log formation constant for the calcium–EDTA complex is 10.70.⁶¹ Thus, the copper complex is favored by 8 orders of magnitude relative to the calcium complex. Conditional formation constants, which describe the effects of pH and other ligands, would be expected to differ by about 8 orders of magnitude as well, and therefore, the possibility of exchanging copper for calcium in the copper–EDTA complex is vanishingly low. To experimentally verify this assertion, we used inductively coupled plasma–optical emission spectroscopy (ICP–OES) to measure the concentration of Ca^{2+} ions in fresh powdered bovine cortical bone soaked with standard PBS solution in the absence and in the presence of 30 mM Cu–EDTA. Our results show the Ca^{2+} concentration in bone to be, within experimental error, essentially the same with and without 30 mM Cu–EDTA ($[\text{Ca}^{2+}] = 155 \pm 16\text{ }\mu\text{g/L}$ without Cu–EDTA and $148 \pm 25\text{ }\mu\text{g/L}$ with 30 mM Cu–EDTA). Therefore, one can unambiguously conclude that the addition of the Cu–EDTA complex to bone does not cause the exchange of copper for calcium in the bone mineral phase.

Since it has been well established that copper chelates have the ability to produce an overall relaxation enhancement in the solid state,^{48–52} we chose to investigate the paramagnetic relaxation effect of Cu^{2+} ions on the ^1H intrinsic spin–lattice (T_1) relaxation times measured in ^{13}C CP MAS NMR experiments on bone tissues for the purpose of accelerating the data acquisition time in SSNMR. The natural abundance ^{13}C Ramp-CP MAS NMR experiments were performed on

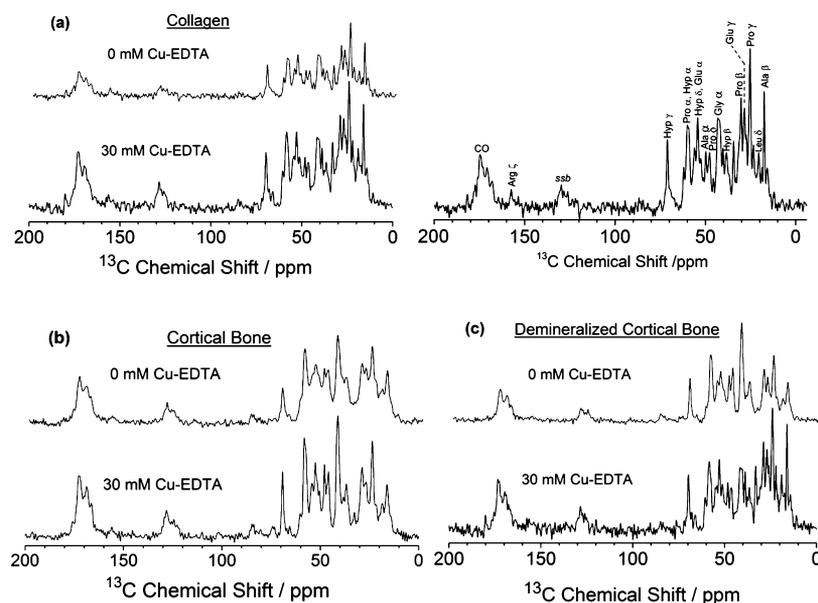


Figure 1. ^{13}C proton-decoupled Ramp-CP MAS NMR spectra of bone samples recorded at a ^{13}C NMR frequency of 226.3 MHz under 10 kHz MAS conditions for (a) type I collagen, (b) powdered bovine cortical bone, and (c) demineralized cortical bone. Samples were soaked in PBS buffer without Cu–EDTA (top) and with 30 mM Cu–EDTA (bottom). Also shown in panel (a) the ^{13}C NMR chemical shift assignments of various amino acids constituting type I collagen in the ^{13}C Ramp-CP MAS spectrum of collagen soaked in PBS buffer. All spectra were recorded at 25 °C on a Bruker Avance 900 MHz solid-state NMR spectrometer equipped with a 4-mm triple-resonance MAS probe. Other experimental parameters include a 2 ms ramp-cross-polarization time, a 75 kHz TPPM proton decoupling during acquisition. Ala, alanine; Leu, leucine; Pro, proline; Glu, glutamic acid; Hyp, hydroxyproline; Gly, glycine; Arg, arginine; CO, carbonyl; *ssb*, spinning sidebands.

powdered samples of type I collagen, fresh bovine cortical bone, and demineralized bone. The ^{13}C chemical shift NMR spectra corresponding to these samples are shown in Figure 1 using an optimized recycle delay after determination of the ^1H T_1 values for type I collagen (Figure 1a), fresh bovine cortical bone (Figure 1b), and demineralized bone (Figure 1c). These spectra were collected without Cu–EDTA (top) and with 30 mM Cu–EDTA (bottom), for which the recycle delays were set to 3 s for the undoped samples and 2 s for the doped ones. It is evident that using the shorter ^1H spin–lattice relaxation times in the presence of 30 mM Cu–EDTA resulted in faster signal acquisition with a noticeable sensitivity enhancement. As expected, the ^{13}C NMR resonances in all of these spectra are dominated by amino acid signals originating from type I collagen. Most of these resonances could be easily recognized and assigned,^{19,31,40} as shown for the collagen ^{13}C MAS spectrum in Figure 1. It is also obvious that no significant variations in the ^{13}C NMR chemical shifts were observed upon addition of 30 mM Cu–EDTA. This indicates that no major structural variations are likely to occur in the investigated bone samples due to the presence of Cu–EDTA.

To obtain a detailed quantitative insight into the paramagnetic effect of copper-chelates on relaxation, the ^1H T_1 values for various amino acid residues were calculated from the data collected by ^1H inversion recovery experiments detected by ^{13}C Ramp-CP MAS. Figure 2 shows the ^{13}C Ramp-CP MAS spectra of the three bone samples acquired at different inversion recovery delays without (a, c, and e) and with (b, d, and f) 30 mM Cu–EDTA. The ^1H T_1 values for different amino acid residues in the investigated samples are significantly reduced by the addition of Cu–EDTA. To illustrate, the series of ^{13}C MAS spectra of type I collagen in Figure 2b show a null signal between 0 and 0.3 s in the presence of Cu–EDTA, whereas the spectra without Cu–EDTA (Figure 2a) display an

almost null signal after 0.5 s; the average ^1H T_1 values determined for collagen from these experiments are (a) 632 ± 49 ms and (b) 218 ± 38 ms. Similarly, the ^{13}C MAS spectra of cortical bone display a null signal between 0.3 and 0.6 s in the presence of Cu–EDTA in Figure 2d, whereas the spectra in the absence of Cu–EDTA show a signal close to null only after 1.0 s (Figure 2c); the average ^1H T_1 values obtained from these experiments for cortical bone are (c) 1174 ± 71 ms and (d) 540 ± 48 ms. For demineralized bone, a near null signal is observed after 0.4 s in the presence of Cu–EDTA (Figure 2f) and after 1.0 s without Cu–EDTA (Figure 2e), thus yielding a calculated average ^1H T_1 values of (e) 1261 ± 63 ms and (f) 394 ± 28 ms for this sample. These numerical results indicate that ^1H T_1 values were successfully reduced by factors of 2.2, 2.9, and 3.2 for bovine cortical bone, type I collagen, and demineralized bone, respectively. By virtue of the paramagnetic Cu–EDTA doping method, these SSNMR measurements can therefore be accelerated up to three times using the shorter ^1H T_1 values, given that the sample characteristics (quantity, stability, etc.) and the duty cycle of the NMR probe are not the impeding elements.

Shown in Figure 3 are the calculated site-specific ^1H T_1 relaxation times for the different amino acids in each of the three materials in the presence of 30 mM Cu–EDTA in comparison with the undoped samples. In the absence of Cu–EDTA, the ^1H T_1 relaxation times are different among protons of a variety of amino acid residues at different positions; these variations of the ^1H T_1 relaxation times are most likely due to internal structural flexibility and relative molecular mobility. In the presence of Cu–EDTA, the average ^1H T_1 relaxation times are considerably shortened, whereas the overall trend for relaxation times as a function of the amino acid residues is essentially preserved. However, a closer examination of the

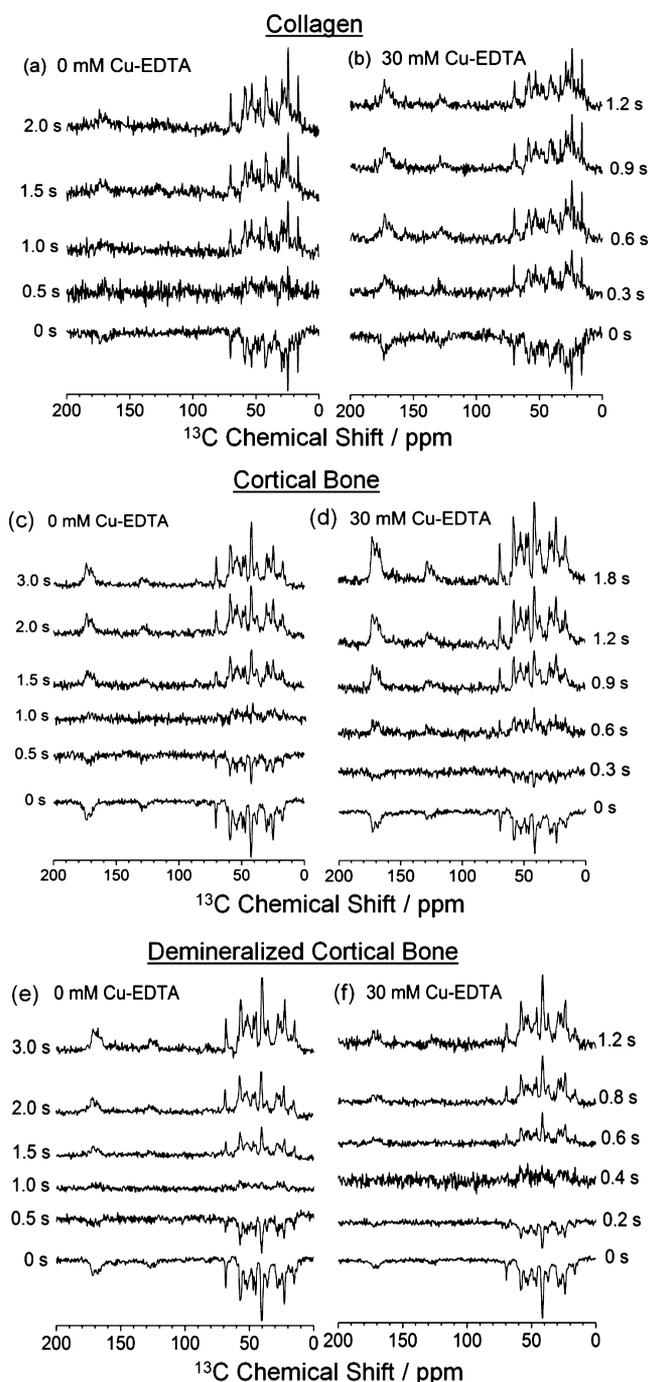


Figure 2. ^1H inversion recovery spectra of ^{13}C Ramp-CP MAS NMR experiments of (a and b) type I collagen, (c and d) cortical bone, and (e and f) demineralized bone. Samples were soaked in PBS buffer (a, c, and e) without Cu-EDTA and (b, d, and f) with 30 mM Cu-EDTA. All spectra were recorded under 10 kHz MAS at 25 °C. Other experimental and data processing details are mentioned in Figure 1 caption. Inversion recovery delays are indicated for each spectrum.

measured ^1H T_1 delays reveals that the shortening in T_1 times is not consistent among all the amino acid residues.

A more detailed analysis of the effect of Cu-EDTA on the ^{13}C line width is given in Figure 4 which shows the ^{13}C line width measured as the full width at half-maximum (FWHM) of the signal from each of the amino acid residues with and without Cu-EDTA. Interestingly, we find that while the presence of 30 mM Cu-EDTA in type I collagen induces a

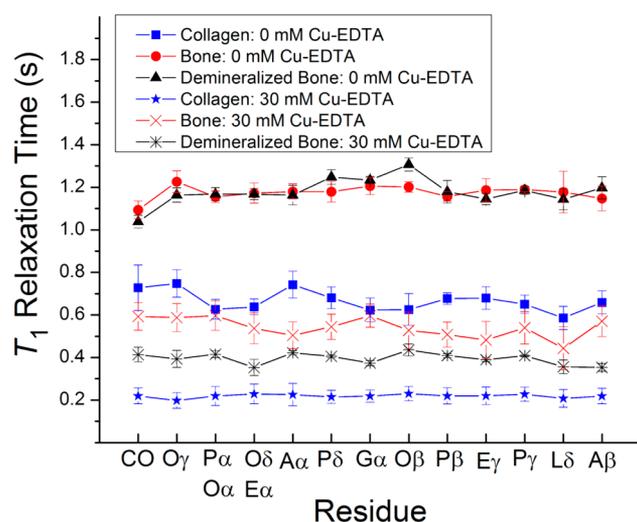


Figure 3. Spin-lattice (T_1) ^1H relaxation times for the various residues in collagen, powdered cortical bone, and demineralized bone in the absence and in the presence of Cu-EDTA (30 mM). The T_1 values were determined from ^1H -spin-inversion recovery experiments detected in ^{13}C Ramp-CP MAS, and the reported errors were estimated from the best-fitting of experimental data. All measurements were performed on a 900 MHz Bruker AVANCE solid-state NMR spectrometer. Other experimental and data processing details are mentioned in Figure 1 caption. A, alanine; L, leucine; P, proline; E, glutamic acid; O, hydroxyproline; G, glycine; CO, carbonyl. The signals from ($P\alpha$, $O\alpha$) and ($O\delta$, $E\alpha$) overlap in the ^{13}C NMR spectrum.

modest increase in the average ^{13}C line width from 370 ± 121 to 409 ± 98 Hz, it has an opposite effect on the average ^{13}C line width in the other two specimens; the average carbon line width in bovine bone decreases from 657 ± 180 to 413 ± 99 Hz while that of demineralized bone decreases from 488 ± 151 to 294 ± 88 Hz upon doping in 30 mM Cu-EDTA. It has been proven that Cu^{2+} has the ability to reduce the T_1 of protons while minimally affecting the ^{13}C line shapes in CP MAS experiments on microcrystalline proteins in the absence of close contact.^{48–50} Even though line-narrowing rather than line-broadening due to Cu-EDTA doping was observed in our case for cortical and demineralized bone samples, further systematic studies of the paramagnetic effect of Cu^{2+} on the ^{13}C spin-spin relaxation times (T_2) are needed to provide more reliable information in that aspect.

Finally, the resolution of observed spectral lines in the ^{13}C CP MAS spectra for cortical bone and demineralized bone was investigated with and without 30 mM Cu-EDTA. Figure 5 shows the aliphatic regions of the ^{13}C CP MAS spectra for cortical bone (a and b) and demineralized bone (c and d) along with the difference between the spectra acquired without and with 30 mM Cu-EDTA for both samples. As mentioned previously, no major changes in the ^{13}C resonance positions were observed by the addition of 30 mM Cu-EDTA. Although line narrowing instead of line broadening due to Cu-EDTA was detected, the intensities of few resonances (denoted by arrows in Figure 5) were significantly reduced in the spectrum of the demineralized bone. In the absence of mineral, the quenching of these signals (namely Gly C_ω , Pro C_δ , and Ala C_α), in which the ^{13}C spins undergo faster spin-spin (T_2) relaxation due to paramagnetic Cu^{2+} proximity, indicates that these residues are more exposed to the bone mineral surface than other residues in the bone organic matrix. Theoretically,

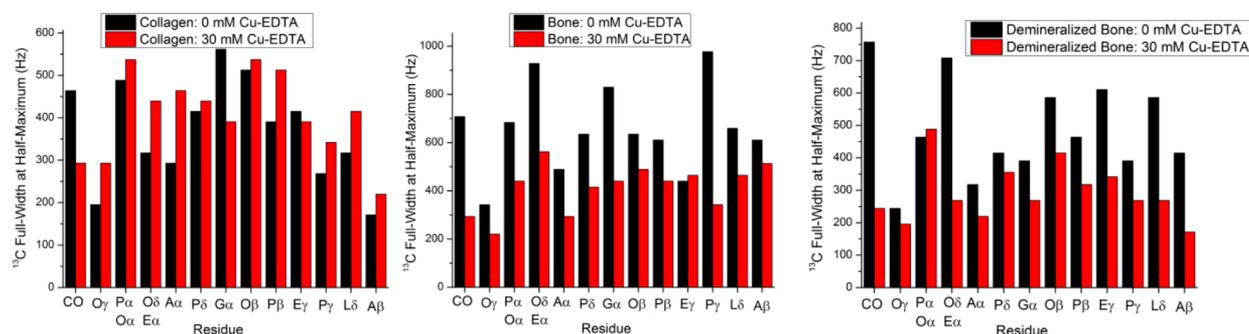


Figure 4. Full-width at half-maximum (FWHM) values obtained from ¹³C Ramp-CP MAS NMR spectra of bone samples recorded at a ¹³C NMR frequency of 226.3 MHz under 10 kHz MAS conditions for type I collagen, powdered bovine cortical bone, and demineralized cortical bone without Cu-EDTA (black) and with 30 mM Cu-EDTA (red). Other experimental and data processing details are mentioned in Figure 1 caption. A, alanine; L, leucine; P, proline; E, glutamic acid; O, hydroxyproline; G, glycine; CO, carbonyl. The signals from (P_α, O_α) and (O_δ, E_α) overlap in the ¹³C NMR spectrum.

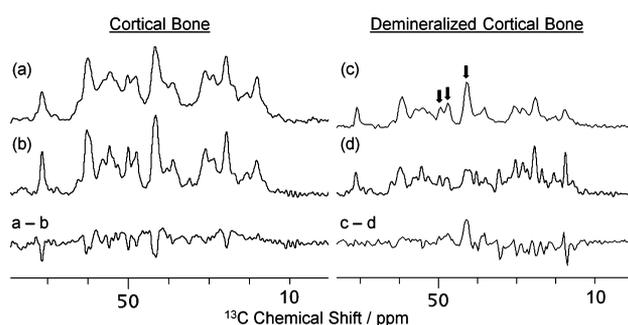


Figure 5. Carbon-13 Ramp-CP MAS NMR spectra of (a and b) powdered cortical bone and (c and d) demineralized bone samples recorded at a ¹³C NMR frequency of 226.3 MHz under 10 kHz MAS without Cu-EDTA (a and c) and with 30 mM Cu-EDTA (b and d). For each sample, the difference between the spectra obtained without and with Cu-EDTA is also shown. Other experimental and data processing details are mentioned in Figure 1 caption.

the observed signal quenching can be attributed to the fact that the paramagnetic T_2 relaxation rates are inversely proportional to r^6 , where r is the distance between the paramagnetic ion and the observed spin.^{53,62} Thus, these residues must correspond to locations of close contact between the mineral and the organic matrix in bone such that in the absence of mineral, Cu²⁺ can come into close proximity of the newly exposed residues. It is therefore plausible that signals for amino acid residues in closer proximity to the bone mineral surface are significantly quenched, while the signals for the majority of other residues remain unaffected by paramagnetic T_2 relaxation enhancement. Further investigations and analyses are required in order to assess the validity of using such enhancements to obtain quantitative structural information from bone samples.

CONCLUSIONS

In conclusion, we have demonstrated that the doping of powdered bone tissues in a copper-EDTA complex results in a 2- to 3-fold enhancement in the intrinsic spin-lattice relaxation rates of protons, which leads to faster data acquisition without affecting the resolution of their ¹³C CP MAS NMR spectra. Although this enhancement is not as drastic as those reported for microcrystalline proteins, it is still significant and will be of ample applicability in structural and dynamic studies of traditionally complex materials like bone. It is worth noting that, although previous solid-state NMR reports have discussed

the use of paramagnetic doping to shorten the proton T_1 values in MAS NMR measurements of crystalline proteins and peptides, the effects of paramagnetic ions on T_1 relaxation times in heterogeneous biomolecular systems have not been tackled in depth. In this study, this approach has been explored in one-dimensional ¹³C Ramp-CP MAS for powdered bones, and it is possible that the same approach can be implemented in CP MAS or stationary NMR experiments on intact bone, as intact bone would give more useful results than the cryogenically milled bone powders used in the present experiments. The successful enhancement of the longitudinal relaxation rates of protons in this study opens up new avenues for sensitivity enhancements for the majority of signals in complex biological systems in more complicated multidimensional SSNMR experiments. Moreover, the combined application of paramagnetic doping and radio frequency pulse sequences tailored specifically for this purpose under much faster MAS rates would also provide further signal enhancements. Finally, a comprehensive study involving comparison of the paramagnetic relaxation effects of various metal-chelates like Cu²⁺, Mn²⁺, and Ni²⁺ and lanthanide ions (e.g., Gd³⁺ and Dy³⁺) on bone samples is currently underway in our laboratory. We strongly believe that such a study can provide additional high-resolution insights on the structure and dynamics in bone and related biomaterials.

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Notes

The authors declare no competing financial interest.

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