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Time-Resolved Dehydration-Induced Structural Changes in an Intact Bovine Cortical Bone Revealed by Solid-State NMR Spectroscopy

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Abstract

Understanding the structure and structural changes of bone, a highly heterogeneous material with a complex hierarchical architecture, continues to be a significant challenge even for high-resolution solid-state NMR spectroscopy. While it is known that dehydration affects mechanical properties of bone by decreasing its strength and toughness, the underlying structural mechanism at atomic-level is unknown. Solid-state NMR spectroscopy, controlled dehydration, and H/D exchange are used for the first time to reveal the structural changes of an intact piece of bovine cortical bone. Proton spectra are used to monitor the dehydration of bone inside the rotor and high-resolution ^{13}C chemical shift spectra obtained under magic angle spinning are used evaluate the dehydration-induced conformational changes in bone. Experiments reveal the slow denaturation of collagen while the *trans*-Xaa-Pro conformation in collagen is unchanged due to dehydration. Our results suggest that GAGs in the collagen fiber and mineral interface may chelate with a Ca^{2+} ion present on the surface of the mineral through sulfate or carboxylate groups. These results provide insights into the role of water molecules in the bone structure and shed light on the relationship between the structure and mechanics of bone.

Water is an important component of cortical bone, accounting for approximately 20% by volume.[1] It is well known that interstitial water molecules play a major role in stabilizing both collagen and mineral through enthalpic stabilization and hydrogen bonding.[2] The water content of bone tissue decreases with age and is associated with a reduction in biomechanical properties that are critical to the function of bone.[3] The loss of water in collagen decreases the toughness of bone and the loss of water associated with the mineral decreases both bone strength and toughness.[4] However, the underlying mechanisms including structural changes in the matrix protein and mineral, and changes in their interactions brought about by dehydration are unclear. Understanding this mechanism could provide insights into the susceptibility of bone to fracture, especially in the osteoporotic tissue of many elderly people. While previous solid-state NMR experiments on bone and related materials have provided valuable structural information,[5–10] an intact bone still remains as a complex system for high-resolution structural studies. In

Supporting Information Available: Assignment and values of ^{13}C chemical shifts, ^{1}H NMR spectra, Raman spectra, and water content evaluation of cortical bone. This material is available free of charge via the Internet at http://pubs.acs.org.

this study, we report the dehydration-induced effects on an intact bovine cortical bone using magic angle spinning (MAS) experiments.

^{13}C chemical shift spectra of a fresh cortical bone, dehydrated cortical bone, and an H_2O/D_2O (H/D) exchanged cortical bone are shown in Fig. 1 along with the resonance assignment from previous studies[11,12]. The dehydrated cortical bone was prepared by placing a piece of cortical bone in a vacuum desiccator for 3 days, while the H/D exchanged cortical bone was prepared by immersing the fresh intact cortical bone in deuterated phosphate buffer for three days. The ^{13}C chemical shift spectrum of a fresh cortical bone exhibits well-resolved spectral lines and is dominated by resonances from collagen (Fig. 1a). The assignment of peaks originating from most amino acids present in the cortical bone matrix is indicated in Fig. 1a (see a more detailed assignment of peaks in Tables S1–S3, Supporting Information). The spectra of a dehydrated cortical bone (Fig. 1b) and a H/D exchanged cortical bone (Fig. 1c) are similar, and exhibit much broader spectral lines than the fresh cortical bone (Fig. 1a). The only major difference between these two spectra (Figs. 1b and 1c) is the disappearance of the peak ~78 ppm representing GAGs due to H/D exchange (Fig. 1c).

Since there are considerable differences between ^{13}C spectra of fresh (Fig. 1a) and dehydrated (Fig. 1b) bones, we performed NMR experiments to study the water-dependent structural and dynamical changes in the intact bone by slowly dehydrating a cylindrical specimen of bovine cortical bone in an NMR rotor. The sample was spun by a constant velocity stream of air against the tip of the rotor, which creates a constant reduced pressure at the distal end, and dehydration of the bone was induced by means of a 0.1 mm diameter hole in the distal end cap of the rotor. At the reduced pressure the water content of the specimen decays exponentially with time (t=7.34 h, Fig. S1) over 24 h. Since the ^{13}C spectrum as shown in Fig. 1 contains well-resolved spectral lines, dehydration-induced changes in bone were monitored as the change in the intensities and line widths of ^{13}C (Fig. 2A). The extent of water in the cortical bone (Fig. S1) was monitored from the decreasing water ^{1}H NMR peak intensity from a series of ^{1}H MAS spectra of the intact bone (Fig. 2B); differences in the ^{1}H NMR spectra of intact cortical bone and ground cortical bone powder are highlighted in Fig. S2. In control experiments no spectral changes were observed even after 24 hours of spinning the sample if the end cap was tightly sealed to prevent evaporation.

The appearance of 3 well-resolved peaks in the carbonyl carbon chemical shift region (~170 ppm) indicates the native collagen structure in a fresh cortical bone is in agreement with a previous study on pure collagen[11]; a ^{13}C MAS spectrum of collagen type I is given in Fig. S3. Interestingly, these well-resolved peaks broaden to a single peak due to dehydration whereas the integrated area of these peaks did not change (Fig. 2A). Peaks in 10–80 ppm region including Hyp Cγ, Hyp C_β, Pro C_α, Gly C_α, Pro C_β, and Pro C_γ peaks gradually decreased in intensity and broadened as the cortical bone was dehydrated with time. Some less intense peaks including Thr C_β at 67.4 ppm, Ser C_β and Val C_α at 62.2 ppm, Val C_β at 33.0 ppm, and Glu C_γ and Lys C_δ at 28.3 ppm disappeared due to dehydration-induced line broadening. The line broadening caused by dehydration can be attributed to a local conformational disorder of the bone matrix and also to the slow dynamics of side chains of collagen.[12] The sensitivity of the $^{13}C_\beta$ chemical shift of Ala to the backbone conformation of collagen can be used to understand the specific structural changes due to dehydration.[12–14] The presence of a 17.6 ppm peak from $^{13}C_\beta$-Ala confirms the triple-helix structure of collagen in the fresh wet cortical bone. Due to dehydration of the bone, the relative intensity of this peak gradually decreased, broadened, and shifted from 17.6 to 18.8 ppm. This observation most likely indicates a change in the triple-helical structure and dynamics of collagen due to dehydration. The observed 5.3 ppm difference between $^{13}C_\beta$ (30.5 ppm) and $^{13}C\gamma$ (25.2 ppm) chemical shifts of Pro of a fresh cortical bone in this study confirms the presence of a *trans*-Xaa-Pro conformation in collagen.[12] Most importantly, the dehydrated bone also exhibited the chemical shift difference value

(Table S2, Supporting Information) suggesting the *trans*-Xaa-Pro peptide bond conformation is maintained during the dehydration process.[15,16] It should be noted that no significant differences were observed in the Raman spectra of these bone samples.

In the ^{13}C NMR spectra of a H/D exchanged cortical bone, the glycosaminoglycans (GAGs) peak at 76 ppm[17] becomes weaker in intensity and almost disappears. The spectra of a dehydrated bone and H/D exchanged bone are similar in the 0–60 ppm chemical shift region but differ from that of the fresh cortical bone. For example, a significant line broadening was observed for Ala $^{13}C\alpha$ at 47 ppm and Ala $^{13}C\beta$ at 17.6 ppm from both dehydrated (Fig. 1b) and H/D exchanged (Fig. 1c) bones, while this effect is greater in the case of the H/D exchanged bone. This observation could be attributed to relatively more structural changes due to the H/D exchange process as D_2O forms weaker hydrogen bonds than H_2O and therefore can disrupt hydrogen bonding leading to structural changes in collagen fiber. The ring carbons of GAG molecules in bone are motionally-restricted due to its rigid pyranose structure. The almost complete disappearance of GAGs peak at 76 ppm indicates a significant disorder in the conformation particularly in the H/D exchanged bone. This observation implies that GAGs in the collagen fiber and mineral interface may chelate with a Ca^{2+} ion present on the surface of the mineral through sulfate or carboxylate groups, while on outside they form hydrogen bonds with surrounding water molecules. H/D exchange disrupts its bonding with water and change its aqueous environment, which further disorders its conformation and cause the line broadening effect in NMR spectra as shown by the reduction in the intensity of the GAGs peak.

Cortical bone is a complex biological system with an intricate structure and unique mechanical properties. It is more meaningful to study it as a whole intact bone than to look its components like collagen or mineral separately. Moreover, the cut large section of bovine cortical bone used in our experiments may give more accurate results than cryogenically milled bone powder commonly used in most previous solid-state NMR studies, especially in experiments involving changing the physical dimensions of the tissue. In this study, we have demonstrated that solid-state NMR experiments on an intact bone can provide high-resolution spectral lines, the effect of dehydration inside the NMR rotor can be monitored, and the observed dehydration-induced spectral changes can provide insights into structural changes in bone. For cortical bone, a highly heterogeneous material with a complex hierarchical structure, high-resolution solid-state NMR can probe selected magnetic nuclei as shown by our results and give snapshot pictures of cortical bone structure at the atomic-level that are difficult to obtain by other methods. Our results further suggest that the use of higher magnetic fields and sophisticated RF pulse sequences under ultrafast MAS can provide more piercing atomic-level structural insights into an intact bone structure that would be useful to understand some unclear structural mechanism about the role of water molecules play in bone structure, toughness and mechanic strength.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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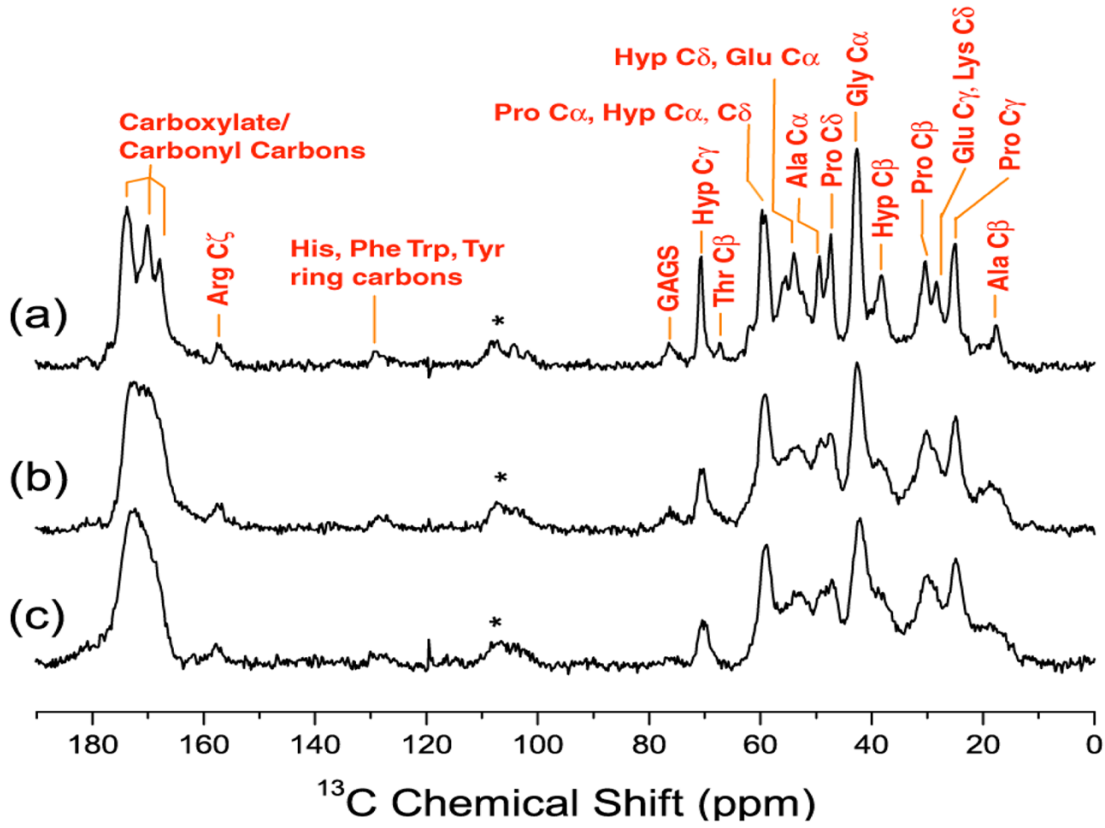

Fig. 1.

Carbon-13 chemical shift NMR spectra of an intact (a) fresh, (b) dehydrated, and (c) D_2O exchanged 60 mg bovine cortical bone. All spectra were obtained using a Varian VNMRS 600 MHz solid-state NMR spectrometer and a 4 mm double resonance MAS probe with a 2ms ramp-cross-polarization, a 80 kHz TPPM proton decoupling during acquisition, and a 3 s recycle delay under 10 kHz MAS at room temperature (25°C). Spinning sidebands are marked with asterisks. The bone samples were shaped to a 2.5×2.5×12 mm size and inserted into a 4mm zirconia MAS rotor (see the Supporting Information).

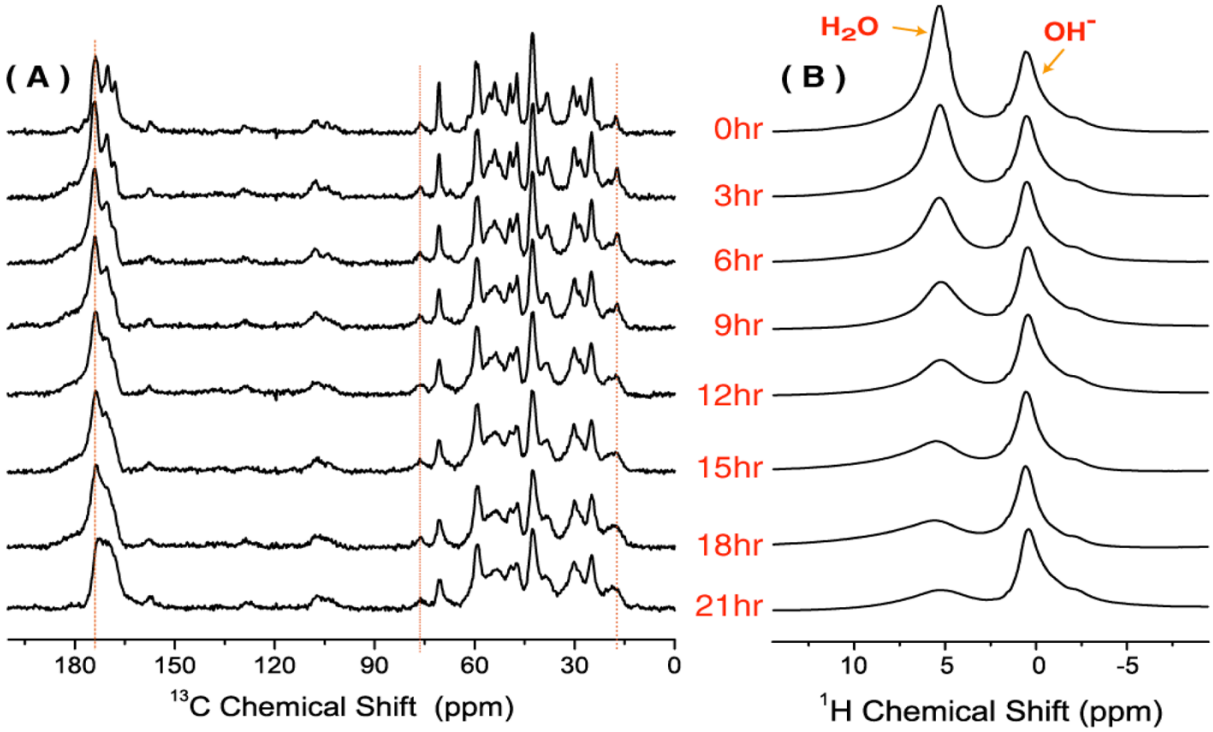

Figure 2.
(A) ^{13}C and (B) ^{1}H chemical shift spectra of an intact bovine cortical bone obtained from a fresh bovine cortical bone (1) and after every 3 hours of dehydration (spectra 2–8). The total acquisition time for each ^{13}C and ^{1}H spectrum was 3 hours and 3 minutes respectively. All other experimental details are as given in Figure 1 caption.