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Selective Detection and Complete Identification of Triglycerides in Cortical Bone by High-Resolution ^1H MAS NMR Spectroscopy

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Abstract

Using ^1H -based magic angle spinning solid-state NMR spectroscopy, we report an atomistic-level characterization of triglycerides in compact cortical bone. By suppressing contributions from immobile molecules present in bone, we show that a ^1H -based constant-time uniform-sign cross-peak (CTUC) two-dimensional COSY-type experiment that correlates the chemical shifts of protons can selectively detect a mobile triglyceride layer as the main component of small lipid droplets embedded on the surface of collagen fibrils. High sensitivity and resolution afforded by this NMR approach could be potentially utilized to investigate the origin of triglycerides and their pathological roles associated with bone fractures, diseases, and aging.

Graphical abstract

Using a ^1H -based constant-time uniform-sign cross-peak (CTUC) COSY-type MAS NMR experiment to selectively detect mobile lipids in compact bone and type-I collagen, we have identified a triglyceride layer as the main component of small lipid droplets embedded on the surface of collagen fibrils.

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Introduction

A substantial body of experimental and clinical data provides evidence on the potential roles played by dietary lipids and lipophilic vitamins in skeletal metabolism and bone physiology, particularly in mineral deposition and osteoblast function.^{1–4} Lipids, which constitute about 2% of the dry weight of bone matrix, reduce the radial permeability of compact bone by influencing the metabolic functions of bone osteoblasts and osteocytes.⁵ Lipids surround the cell body and control the transport and exchange of nutrients and other signaling molecules between the cell and its environment.⁴ Nonetheless, how these postprandial lipoprotein constituents are actually delivered to bone cells *in vivo* and how these molecules are structured and distributed in the extracellular bone matrix has been a topic of considerable scientific interest. In fact, the existence of a probable association between lipids and biomineralization was suggested several decades ago.⁶ Bone, a heterogeneous biocomposite material with remarkable properties, is composed of a soft organic matrix (primarily type-I collagen along with several non-collagenous proteins, lipids, polysaccharides, and citrates) reinforced with an inorganic rigid mineral (mainly poorly crystalline carbonated hydroxyapatite-like nanocrystals).^{3,7} An atomic-level investigation of the structural organization of the various organic and inorganic constituents in bone, along with their associated interactions, would thus provide valuable insights into the material properties and potentially into the molecular pathway of bone formation and metabolism. Such studies, however, have proven to be difficult because the complex heterogeneous nature of bone poses significant challenges to the application of most commonly used biophysical techniques. In addition, traditional chemical methods are potentially disruptive, can cause perturbations in bone ultrastructural features, and generally do not provide molecular-level information.⁸ While solid-state NMR spectroscopy is a well-established non-destructive technique capable of studying bone and other biomineralized tissues without any chemical pre-treatment, poor resolution of the ¹H NMR spectra, caused by strong dipolar couplings among protons in addition to the appearance of a predominant water signal, has been a major impediment for obtaining high-resolution structural information from bone tissues using ¹H-based NMR methodologies. Thus, applications of solid-state NMR so far have generally been focused on the observation of ³¹P and ⁴³Ca nuclei in the bone mineral and ¹³C nuclei in bone proteins.^{8–23} Owing to the remarkable advances in NMR probe technology, a recent ¹H NMR study on bone performed under 100 kHz ultrafast magic angle spinning (MAS) conditions provided better resolution, which illustrates the potential to investigate the bone proteins by proton-based ultrafast MAS NMR techniques.²⁴

In addition to proteins, a considerable quantity of triglycerides (TG) and cholesterol esters (CHE) was identified by the analysis of the extracts from bone.^{4,5,25} It should be noted that bone is a dynamic tissue that undergoes continuous remodeling; hence its lipid content is not fixed, but varies with factors such as age, species, tissue location, diet and health.⁴ Since the physiological role of lipids has been a subject of considerable research, it is therefore timely and essential to obtain high-resolution information on the particular lipid composition and the distribution of lipids in bone. A comprehensive AFM (atomic-force microscopy) study on the cortical bone of bovine tibia showed that a layer of round lipid particles cover the collagen fibrils, which explains the relatively high concentration of these apolar lipids in

cortical bone;²⁶ however, the exact components of these lipids were not clearly identified in that study. Recently, Reid *et al.* used NMR spectroscopy to identify fatty acyl-rich lipids in the minerals from three calcified tissues (intimal vascular, medial vascular, and human and equine bones),²⁷ in which the relative abundance of methyl-substituted fatty acids might indicate that they are residuals of lipoprotein particles and not of phospholipid membranes. In the current study, we show that a magic angle spinning (MAS) ^1H -based constant-time 2D NMR experiment can be used to selectively detect and characterize triglycerides present in cortical bone by taking advantage of the dynamical differences among various molecular species for suppressing signal contributions from other components of bone. This atomic-level characterization of triglycerides from the complex bone matrix is critical for understanding the role played by triglycerides and lipids in bone metabolism, and thus enables high-resolution structural studies of the extracellular bone matrix.

Results and discussion

To identify the type of lipids present in the extracellular matrix, a COSY-type experiment using constant-time uniform-sign cross-peak (CTUC) COSY sequence was carried out on bovine cortical bone, whose spectrum is shown in Fig. 1c. This ^1H -detected CTUC COSY-type sequence is a modified version of the corresponding ^{13}C NMR sequence achieved by removing the CP portion (Fig. 1b).^{28,29} The constant-time COSY experiment offers much higher resolution than the normal COSY experiment for systems with relatively short T_2 transverse relaxation times.²⁹ The assignment of proton peaks was performed using this COSY-type spectrum along with a ^1H - ^1H double quantum-single quantum (DQ-SQ) spectrum (Fig. 1d) that shows pairs of correlation peaks arising from pairs of nearby protons observed in the single-quantum (SQ) dimension, and are located at the sum of their isotropic NMR chemical shifts in the double-quantum (DQ) dimension. Such spectrum provides specific information on proton-proton proximities (up to 3 Å) within the molecular framework, which in turn describes the molecular-level organization in the system. Based on the connectivity observed in the COSY-type spectrum (Fig. 1c) and comparing with the ^1H NMR spectrum of a solution sample of triglyceride,^{30–32} we can infer that the sharp ^1H peaks observed from cortical bone should arise from triglyceride lipid; the numbering of carbons in triglyceride and fatty acids is shown in Fig. 1a while the assignment ^1H peaks is shown in Fig. 2c and 2d).

In comparison to the spectrum of cortical bone shown in Fig. 1c, the 2D CTUC COSY-type spectrum of type-I collagen shows similar features, albeit with the appearance of few additional peaks (Fig. S2, ESI). Triglyceride is one of the main components in serum lipoproteins; however, the concentration of triglyceride in bloodstream is very low.³³ Therefore, the serum lipoproteins are not the main source of the high intensity triglyceride peaks observed in the ^1H NMR spectra of cortical bone. Since triglyceride also exists in pure collagen, the observed triglyceride must be a part of the mobile lipid particles on the surface of the collagen fibrils. This is consistent with the AFM study on the cortical bone of bovine tibia that showed a layer of round lipid particles covering the collagen bundles.²⁶ Importantly, the narrow ^1H NMR spectral lines observed in our spectra also suggest that the triglyceride in the lipid droplets must be highly mobile on the NMR time scale.

Fig. 2 shows the ^{13}C NMR spectra of cortical bone and type-I collagen. It is evident from the ^{13}C MAS NMR spectra acquired by ^1H -to- ^{13}C cross-polarization (CP), which selectively enhances the ^{13}C signals from rigid and immobile molecules, that most carbon signals in cortical bone arise from collagen. However, the resolution of ^{13}C CPMAS spectrum of cortical bone is better than that of collagen, which is likely due to the dynamic effects of collagen under different hydration levels.³⁴ Of note is that signals from non-collagenous proteins and other macromolecules present in the bone matrix are obscured by the strong signals from various type-I collagen amino-acid residues that can be readily assigned based on previously reported values.^{19,22,35} On the other hand, the ^{13}C signals from the highly mobile molecules in cortical bone and collagen can be detected using the RINEPT (refocused insensitive nuclei enhanced by polarization transfer) NMR experiment,^{36–38} as depicted by the bone spectrum in Fig. 2c. This high-resolution spectrum indicates that the only highly mobile molecules in cortical bone are triglycerides, by comparing with the typical spectrum of triglyceride.^{30,39,40} This is also consistent with the NMR analysis of Schulz *et al.* that revealed that signals from highly mobile triglycerides, and not from phospholipids, dominate the directly polarized ^{13}C MAS NMR spectra of native rabbit bones and implants.⁴⁰ The ^{13}C peak assignment in the one-dimensional RINEPT spectrum was achieved by the 2D ^{13}C - ^1H heteronuclear chemical shift correlation (HETCOR) spectrum using RINEPT polarization transfer under MAS; the 2D correlation spectrum recorded on bone along with the corresponding assignments is shown in Fig. 2d. It should be noted here that a quantitative analysis of the ^{13}C MAS RINEPT experiment in solids may not provide reliable information due to non-uniform signal enhancements caused by different ^1H - ^{13}C scalar couplings.

To further confirm the lipid component in bone, the bovine cortical bone powder and collagen fibrils were incubated overnight in 2:1 (v/v) chloroform:methanol, and solution-state NMR spectra were collected on the extracts obtained from both materials. The ^1H and ^{13}C NMR spectra of the extracts from cortical bone and type-I collagen are dominated by the triglyceride signals, as shown in Fig. 3 and Table 1. It is clear that no signals from phospholipids, the main lipid in membrane, are observed, which confirms that the lipid contribution from blood vessels is very low (or negligible) and can be ignored. NMR signals from phospholipid and cholesterol are not detected in cortical bones, which indicates that these lipids might come from bone marrow or blood vessels that are abundant in trabecular bones. The ^1H peak integrals for the extracts of cortical bone and collagen show that the chemical structure of the triglyceride in both extracts are similar, as shown in Table 1. By comparing the ratio of proton site 10 to proton site 3 (or 1, 2), for instance, it can be estimated that the relative abundance of free fatty acids (FFA) to TG in both extracts is roughly the same. The liquid-state ^{13}C NMR spectrum of the extract in Fig. 3c is similar to the ^{13}C RINEPT spectrum recorded on a solid cortical bone sample (Fig. 2c), except for the additional carbonate carbon peak at *ca.* 173 ppm, which cannot be detected using the RINEPT polarization transfer method. Moreover, since the ^{13}C NMR relaxation times are much longer than those of protons, the ^{13}C NMR spectrum recorded here with a short recycle delay does not provide reliable quantitative integral analysis. Of note is that ^{13}C solid-state RINEPT experiments were also performed on incubated bone and collagen

samples under 10 kHz MAS, but no signals were observed, which confirms the removal of the triglyceride upon incubation with chloroform:methanol.

Conclusions

In conclusion, we have demonstrated the strong potential of high-resolution ^1H -based NMR spectroscopy for the selective detection and complete identification of triglycerides in complex bone matrix at the atomic level. Our results suggest that the main components of the round lipid particles that cover the collagen fibrils are triglyceride droplets. If the detected triglycerides come from the lipoproteins, which means that lipoproteins undergo fast NMR motions, then the cholesterol esters in lipoprotein could also be detected using the ^1H and ^{13}C NMR experiments used here. However, it is still possible that lipoproteins with low concentrations exist in the lipid particles, but are undetectable by solid-state NMR because of their slow motion on the NMR time scale. Despite that our present findings enable high-resolution structural studies of composite mineralized tissues; many open questions regarding the source and origin of this triglyceride content in bone as well as its exact role in bone mechanical and metabolic functions remain to be addressed. A thorough understanding of the origin and role of these lipids is essential for potential treatments of bone metabolic diseases and disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(a) Molecular fragments of triglyceride and fatty acids, together with the carbon labels. (b) ^1H -detected CTUC 2D COSY-type NMR pulse sequence. The solid and blank rectangles denote $\pi/2$ and π pulses, respectively. (c) 2D $^1\text{H}/^1\text{H}$ NMR chemical shift correlation spectrum of bovine cortical bone recorded at 10 kHz MAS rate using the pulse sequence shown in (b). (d) 2D $^1\text{H}/^1\text{H}$ NMR DQ-SQ correlation spectrum of bovine cortical bone at 10 kHz MAS rate.

Fig. 2.

Carbon-13 CPMAS NMR spectra of (a) bovine cortical bone and (b) type-I collagen obtained using a 2-ms contact time and 10,000 scans. The ^{13}C NMR chemical shift assignments of various amino acids in bone are shown with the three letter code. (c) ^{13}C MAS NMR spectrum of bone obtained using RINEPT polarization transfer. (d) 2D $^{13}\text{C}/^1\text{H}$ correlation spectrum of bone using RINEPT polarization transfer. The time delays τ_1 and τ_2 in RINEPT were 1.8 and 0.8 ms respectively. All spectra were acquired at 10 kHz MAS. The peak assignments in (d) are also indicated using the same labels as those in Fig. 1a.

Fig. 3.

^1H NMR spectra of the extracts released after overnight incubation in 2:1 chloroform:methanol from type-I collagen (a) and bovine cortical bone (b). (c) ^{13}C liquid-state NMR spectrum of the same extract from cortical bone. The ^{13}C peak from CDCl_3 solvent is indicated by an asterisk. All spectra were recorded using a single-pulse excitation. 3s recycle delay and 32 scans were used to acquire ^1H spectra, while 1s recycle delay and 256 scans were used to obtain ^{13}C spectra.

Table 1¹H NMR Chemical Shifts and Quantification from Bone and Collagen Extracts.

Proton ^a	Chemical Shift (ppm)	Bone Extract Integral	Collagen Extract Integral
TG13	5.31	0.45	0.48
TG3	5.23	0.16	0.12
TG1	4.25	0.33	0.25
TG2	4.1	0.33	0.30
CHE	3.68	0.17	0.06
TG11	2.73	0.01	0
TG5	2.27	1	1
TG12	1.97	1.15	0.97
TG6	1.57	1.18	1.14
TG7,8,9	1.26-1.22	10.83	11.56
CHE	0.97	0.05	0
TG10	0.84	1.55	1.71
CHE	0.64	0.03	0
CHE	0.03	0.01	0

^aTG = triglyceride; CHE = cholesterol ester