The Organic−Mineral Interface in Bone Is Predominantly Polysaccharide

Erica R. Wise,[†] Sergey Maltsev,[‡] M. Elisabeth Davies,[§] Melinda J. Duer,[†] Christian Jaeger,[‡] Nigel Loveridge,[||] Rachel C. Murray,[⊥] and David G. Reid*[,†]

Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K., Federal Institute of Materials Research and Testing (BAM), Richard Willstaeter Str. 11, D-12489, Germany, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, U.K., Department of Medicine, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QQ, U.K., and Animal Health Trust, Lanwades Park, Newmarket, Suffolk CB8 7UU, U.K.

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Bone comprises organic and inorganic components in a complex composite which confers remarkable ability to withstand mechanical loading, adapt to the environment, and act as a mineral reservoir.[1] Toughness and stiffness are supplied by the organic and mineral phases, respectively. The former is a matrix of proteins, mainly collagen, and other macromolecules including proteoglycans (PGs) rich in acidic glycosaminoglycans (GAGs). The latter is a hydroxylated calcium phosphate resembling the mineral hydroxyapatite. Although the relationship between the two phases must be crucial to the properties of bone in health and disease, little is known about the macromolecules which constitute and stabilize the boundary. The molecular interaction between these components is fundamental for strength, adaptation, and growth and will be key to new understanding of bone health problems; current literature is dominated by an assumption that it is proteins which stabilize the interface.[2,3] However, in this communication we present a direct demonstration using solid-state NMR (SSNMR) that normal bone mineral contacts its organic matrix via polysaccharides, most likely GAGs in PGs.

As a result of the abundance of ^{31}P nuclei within the mineral phase, the rotational echo double resonance (REDOR) SSNMR technique offers a unique probe of the atomic level structure and composition of the interface.[4] REDOR reintroduces the ^{31}P−^{13}C through-space internuclear magnetic dipolar coupling which is removed by high-speed magic angle spinning (MAS) in standard SSNMR experiments. The magnetic dipolar coupling strength is an inverse cubic power function of internuclear distance and falls off sharply with increasing interatomic separation. Reintroducing this interaction with a train of ^{31}P pulses results in dephasing and loss of signal intensity of those nuclei in proximity to phosphorus. In practice, signals from ^{13}C nuclei less than 5−6 Å away from phosphorus nuclei in the mineral decrease in intensity over recoupling periods of about 10 ms; nuclei closest to the mineral dephase most rapidly.

The signals affected at the shortest dephasing times in ^{13}C{^{31}P} REDOR experiments[5] are the distinct shoulder on the high-frequency edge of the carbonyl/carboxylate signal, at approximately 182 ppm, a lower frequency element of this envelope at approximately 175 ppm, and a broad signal centered at 76 ppm (Figure 1A).

At longer dephasing times, small effects, which are nevertheless highly reproducible between adult samples, are seen on several signals, most of which are probably from protein, including collagen. Most of the signals in the ^{13}C spectrum can be assigned by reference to the chemical shifts of the amino acid residues constituting Type I collagen;[6,7] a standard cross-polarization (CP) MAS spectrum appears as Figure 1B with some assignments. While the 182 and 175 ppm signals are consistent with carboxylate/carbonyl carbons which could belong to proteins, no common amino acid gives rise to chemical shifts which approximate that of the strongly dephasing 76 ppm signal. It must therefore arise from a non-protein biomolecule or protein carbons in a magnetically unusual environment or one affected by post-translational modification. It is unlikely to be from the γ-carbons of the hydroxyproline (71 ppm), which is abundant in collagen, or γ-carboxyglutamate (55 ppm), which occurs in several other bone proteins like osteocalcin. Nor can it be the β-carbon of phosphoserine (61 ppm) found in other bone phosphate carrier proteins or any other covalently phosphorylated carbon, because the REDOR dephasing to such a carbon would be much more rapid than we observe.[4,8] However, it is consistent with some of the secondary alcohol carbons in pyranose sugars like those comprising the oligosaccharide component of PGs or sialoproteins.[7,9]

Connective tissue GAGs consist of variants of a few primary structural types: hyaluronic acid, keratan, and chondroitin, which are often sulfated, and dermatan. Bone SSNMR signals are too broad to distinguish these species, and the REDOR-dephasing macromolecules may be of one or several GAG types, sialic acids, other sugars, or a combination of all three.

We have therefore examined the NMR spectra of related tissues which contain high proportions of GAG PGs to assign the strongly dephasing bone signals. Figure 2 compares CPMAS NMR spectra of adult equine subchondral bone with that of equine mineralized cartilage, which contains mineral essentially identical to that of bone by powder X-ray

* Corresponding author. E-mail: dgr30@cam.ac.uk.
[†] Department of Chemistry, University of Cambridge.
[‡] Federal Institute of Materials Research and Testing.
[§] Department of Veterinary Medicine, University of Cambridge.
[||] Department of Medicine, University of Cambridge.
[⊥] Animal Health Trust.

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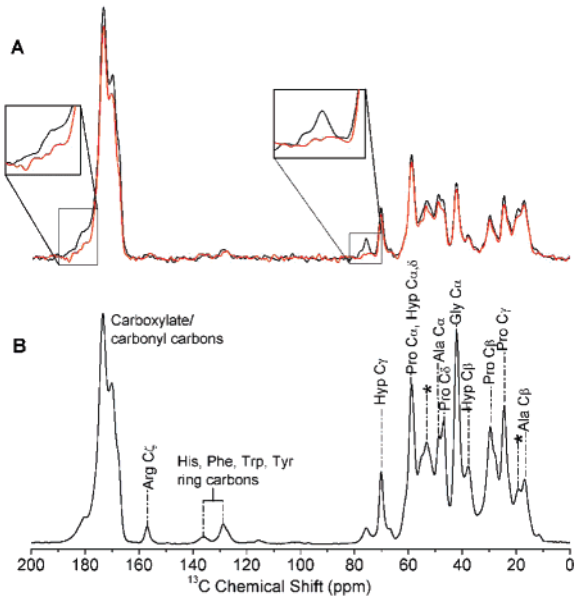

Figure 1. A. Comparison of bone spectra acquired without (black) and with (red) $^{13}C\{^{31}P\}$ REDOR applied for 9.6 ms (MAS rate 12.5 kHz, ^{31}P π pulses applied every 80 μs); the insets highlight the profound dephasing of the 76 and 182 ppm signals. B. Some assignments of the collagen component of the bone spectrum.

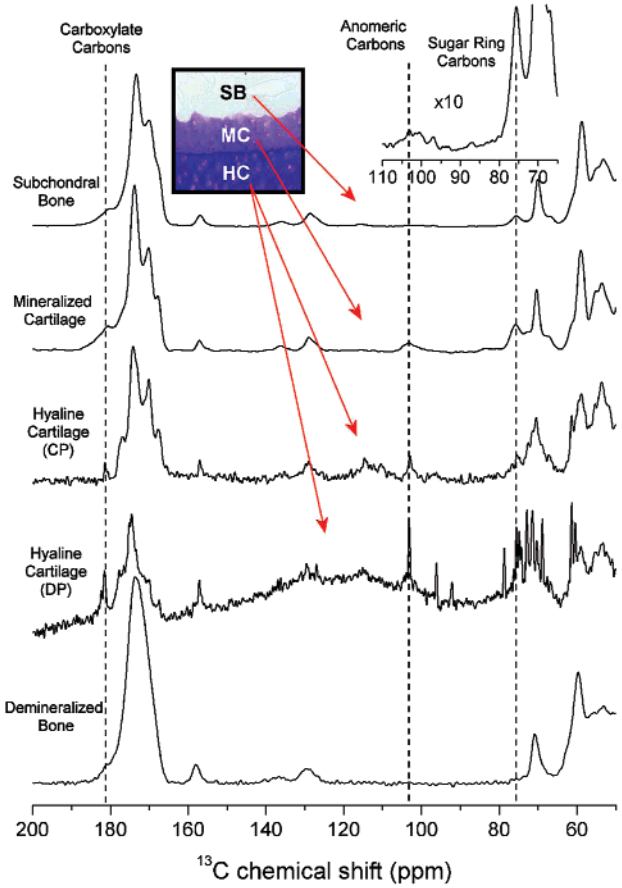

Figure 2. A comparison of the spectra of bone and closely related biomaterials; vertical dotted lines connect signals ascribed to GAG. The inset shows a histological section through a joint stained with the GAG-specific reagent toluidine blue (SB − Subchondral bone, MC − Mineralized cartilage, HC − Hyaline cartilage). Residual signal in the high-frequency 182 ppm shoulder on the carbonyl/carboxylate envelope in the demineralized bone is from protein carboxylate carbons.

diffraction and indeed gives rise to very similar $^{13}C\{^{31}P\}$ REDOR effects. The 76 and 182 ppm signals are relatively more intense in mineralized cartilage than in bone, while another at approximately 103 ppm, broad but nevertheless observable in bone, is also more prominent in mineralized cartilage. Like 76 ppm, 103 ppm is not reconcilable with any of the common amino acids, but is a good match to the resonances of anomeric carbons in β-glycosidically linked polysaccharides. Articular hyaline cartilage shows two families of signals, one broad due to motionally restricted molecules and detectable by CPMAS and the other sharper due to more mobile components and only seen with direct polarization (DP) MAS. Both populations show the signals at 76, 103, 175, and 182 ppm, which are consistent with GAGs like the chondroitin sulfates.[9] The relative prominence of the GAG signals increases from bone, in which GAG is least abundant, through mineralized cartilage, to hyaline cartilage, in which it is most plentiful. If the 76 ppm signal were an amino acid resonance strongly shifted by mineral binding, its intensity would scale with that of the other protein signals and not with the 103 ppm sugar anomeric signal. It should also become more, not less, prominent in bone than it is in mineralized cartilage. Neither is the case. Thus the signal at 76 ppm is from sugars, and consequently REDOR demonstrates that the biomolecules most intimately associated with bone mineral are sugars, quite probably GAGs, and not proteins. Finally, Figure 2 shows part of a spectrum of bone material which has been demineralized by prolonged soaking in ethylenediamine tetraacetic acid; it is essentially like that of whole bone with some signal broadening, except that the GAG signature signals at 76 and 103 ppm are no longer observed. We suggest that demineralization frees soluble GAGs from their natural, intimate association with the mineral phase, at which point they are free to diffuse out of the organic matrix.

In vivo the collagen fiber scaffold directs bone mineralization[1,2] to periodic gaps which accommodate plate shaped mineral particles about 25−35 nm long and wide and 2.5−3.5 nm thick. Later, more mineral inserts between the collagen bundles, so it is natural to inquire about any possible direct interaction of collagen itself with nascent mineral. We have performed $^{13}C\{^{31}P\}$ REDOR on a single sample of equine foetal bone at 6 months gestation and notice that even at long $^{31}P-^{13}C$ recoupling times the only dephasing signals are the familiar ones at 76 and 182 ppm, and others around 48 ppm which could be from ring carbons next to nitrogen in sugar amides and which also dephase consistently in adult bone. We see no effects to other signals ascribable to protein. Although this needs confirmation on more samples, we propose it reflects young mineral which only contacts GAGs, formed in the gap zones of the collagen network. Later in life, mineral inserts in the interstices between the collagen fibrils, and $^{13}C\{^{31}P\}$ REDOR dephasing of both GAG and (more weakly) collagen signals occurs as seen in Figure 1.

Mineralization is influenced by proteins, especially some which are rich in serine (often phosphorylated) and acidic residues.[10] Many, like the small leucine-rich repeat proteins (SLRPs), are glycosylated, but the polysaccharide components have received much less attention in the literature than the polypeptides to which they are attached. In general proteins are uppermost in theories about biopolymer-directed

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mineralization, in spite of multifarious evidence—genetic,[11] in vitro,[12] biochemical,[13] and immunohistochemical[14]—that GAGs are important in bone formation, with a vital role in modulating mineral size and crystallinity. Our SSNMR demonstration that the biomolecules most closely bound to bone mineral are polysaccharides confirms this implicit central role in ensuring ordered biomineralization. In bone, SLRPs bearing long chondroitin and dermatan sulfate chains can also bind to collagen,[15] perhaps orienting GAG chains, and thus ultimately mineral growth, relative to collagen fibrils in nascent bone.

Macromolecules[13,16] modulate biomineralization by directing formation of amorphous inorganic phases and preventing uncontrolled crystallization[17,18] which predisposes to bone weakness.[19,20] Thus moderating crystallization is a vital element of the formation of healthy bone, a process to which the GAGs could be key. Many GAGs adopt regular secondary structures (like the extended ribbon-like conformation of the calcium salt of chondroitin sulfate A[21]) which present numerous recognition points for influencing the growth of hydrated calcium phosphates from dissolved parent ions. Polysaccharide sulfates and carboxylates can chelate Ca^{2+}, and N-acetylamido and hydroxyl groups can hydrogen bond with protonated PO_4^{3-}, water, and OH^-. Thus the GAGs seem better suited to the orderly propagation of the mineralization process than hydrophobic collagen or small globular proteins.

This demonstration that sugars, not proteins, form the interface between the organic and mineral components fundamentally alters an accepted concept of bone structural biology. It provides a unifying rationalization for the diverse influences of GAGs and PGs on calcium phosphate mineral formation in native bone and in vitro and implicates them in bone disease pathogenesis. This could exert a major impact on the pharmacological management of bone disorders by directing novel therapeutic approaches, as it suggests new molecular targets for drug discovery, perhaps based on modulation of GAG metabolism. It also offers new disease biomarkers for diagnosis.

In the more immediate future this discovery will redirect strategies for designing osteomimetic materials[22] and broaden thinking about the design of nanocomposite hard tissue replacement materials, currently dominated by concepts based on proteins[23] partnering calcium phosphate mineral.[24] The chemically and conformationally versatile polysaccharides will influence the design of new useful biocomposites as, or more, fruitfully than ideas based on protein structures. Indeed, synthetic strategies starting from assumptions that proteins regulate normal bone biomineralization seem to be undergoing a process of "convergent evolution" toward scaffolds which, in terms of the chemical functionalities they present to solidifying mineral—abundant carboxylate, hydroxyl, and secondary amide groups—are starting to resemble GAGs more than polypeptides.

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