Contrasts between organic participation in apatite biomineralization in brachiopod shell and vertebrate bone identified by nuclear magnetic resonance spectroscopy

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Unusually for invertebrates, linguliform brachiopods employ calcium phosphate mineral in hard tissue formation, in common with the evolutionarily distant vertebrates. Using solid-state nuclear magnetic resonance spectroscopy (SSNMR) and X-ray powder diffraction, we compare the organic constitution, crystallinity and organic matrix–mineral interface of phosphatic brachiopod shells with those of vertebrate bone. In particular, the organic–mineral interfaces crucial for the stability and properties of biomineral were probed with SSNMR rotational echo double resonance (REDOR). *Lingula anatina* and *Discinisca tenuis* shell materials yield strikingly dissimilar SSNMR spectra, arguing for quite different organic constitutions. However, their fluoroapatite-like mineral is highly crystalline, unlike the poorly ordered hydroxyapatite of bone. Neither shell material shows $^{13}\text{C}\{^{31}\text{P}\}$ REDOR effects, excluding strong physico-chemical interactions between mineral and organic matrix, unlike bone in which glycosaminoglycans and proteins are composited with mineral at sub-nanometre length scales. Differences between organic matrix of shell material from *L. anatina* and *D. tenuis*, and bone reflect evolutionary pressures from contrasting habitats and structural purposes. The absence of organic–mineral intermolecular associations in brachiopod shell argues that biomineralization follows different mechanistic pathways to bone; their details hold clues to the molecular structural evolution of phosphatic biominerals, and may provide insights into novel composite design.

Keywords: *Lingula anatina*; *Discinisca tenuis*; francolite; hydroxyapatite; fluoroapatite; bone

1. INTRODUCTION

Greater understanding of how biological tissue undergoes and maintains mineralization is of major importance to many fields. Successful production of cartilage, bone and dental grafts remains a challenge in tissue engineering [1,2]. Moreover, inappropriate mineralization is a common pathological occurrence, arguing a need for novel therapeutic approaches for its prevention [3]. In previous work we suggest that, in vertebrate biomineral, glycosaminoglycan polysaccharides (GAGs) are prominent at the interface between

bone mineral and the organic scaffold in which the mineral is embedded [4,5]. This observation implies that GAGs participate in the biomineralization mechanism, which seems to be highly conserved across vertebrate species. The availability of calcium phosphate biomineral from organisms that are evolutionarily distant from the vertebrates gives us an opportunity of investigating whether these mechanisms are universal or whether different biomineralization strategies have evolved independently.

To examine the composition and properties of the organic scaffold and mineral, we use solid-state nuclear magnetic resonance spectroscopy (SSNMR) [6] and

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X-ray diffraction. SSNMR is among the most powerful techniques for studying the atomic- and molecular-level structure of pure organic and inorganic substances, minerals, polymers, mixtures and materials. It can be used effectively to investigate poorly ordered or disordered materials, such as most biological tissues and composite materials that resist detailed analysis by conventional chemical and biochemical techniques. Briefly, atoms of certain isotopes of most elements possess the property of nuclear spin. When placed in a strong magnetic field, these atomic nuclei can be thought to assume certain orientations with respect to this field. When irradiated at particular radiofrequencies, these orientations change and the atomic nuclei absorb energy and subsequently emit it. Usually it is this emission which gives rise to the nuclear magnetic resonance (NMR) signals that are measured in an NMR experiment. A great strength of NMR is the fact that each atomic nucleus in a distinct molecular environment gives rise to only a single NMR signal, and the frequency of this signal depends on the chemical and physical environment of the atom; in other words on the molecular framework surrounding it. Thus, the resulting spectrum of signals can provide a unique fingerprint of a given material. Most biological elements possess at least one isotope that is NMR-active, opening numerous possibilities for studying the molecular properties and distribution of that element in biomaterials.

In phosphatic biominerals, we are particularly fortunate in that most of the NMR-active isotope of phosphorus, ^{31}P , is confined to the mineral phase, and most of the NMR-active isotope of carbon, ^{13}C , to the organic matrix in which mineral particles are embedded. (Conveniently, inorganic carbonate ion substituted into the apatite lattice is easily recognized by its characteristic NMR resonance frequency of *ca* 168 ppm.) This distinctive distribution of ^{31}P and ^{13}C to the mineral and organic phases, respectively, presents an attractive opportunity to investigate the composition of the mineral–organic interface. Again briefly, this is because magnetically active atomic nuclei that are close to each other in space (in practice separated by less than *ca* 1 nm) interact, a phenomenon referred to as the internuclear dipole–dipole interaction. Under many circumstances this interaction is a nuisance because it introduces excessive signal broadening that degrades spectral resolution; it is usually controlled using techniques known as magic angle spinning (MAS) and broadband decoupling. However, there is a family of SSNMR techniques that selectively reintroduce internuclear dipole–dipole interactions in a way which can yield detailed information about internuclear distances, and hence molecular structure and intermolecular relationships. Among these is rotational echo double resonance (REDOR) [7,8]. In $^{13}\text{C}\{^{31}\text{P}\}$ REDOR, it is the ^{13}C – ^{31}P internuclear dipole–dipole interaction that is reintroduced by applying radiofrequency pulses at the frequency of ^{31}P , while observing how this changes the ^{13}C spectrum. In ideal cases such as crystalline solids with isolated ^{13}C – ^{31}P ‘spin pairs’, the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR output can be analysed to extract interatomic distances accurate to within less than 0.1 nm. In more complex materials such as

biominerals, such accurate determination of interatomic separations is impossible, and indeed meaningless given the multitude of interacting nuclei; generally, however, atoms will have to be within *ca* 1 nm of each other for the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR effect between them to be significant. In reality there is no sharp ‘cut-off point’, but failure to observe the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR effect can be confidently interpreted as the bulk of carbon populations and phosphorus populations being separated by at least *ca* 1 nm.

We have used $^{13}\text{C}\{^{31}\text{P}\}$ REDOR to show that acidic GAGs are the molecules most strongly associated with the interface in bone [4], dentine [9], mineralized articular cartilage [5] and pathologically calcified atherosclerotic plaque [10]. The apparent ubiquity of the GAG–mineral association has led us to hypothesize that GAGs, with their diversity of polar and charged functional groups, have been naturally selected as widespread, if not universal, participants in calcium phosphate biomineralization.

In vertebrate hard tissue, poorly crystalline hydroxyapatite-like $(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)$ calcium phosphate mineral predominates. Among invertebrates, calcium carbonates are most commonly used to form skeletal materials, although at least 20 invertebrate phyla produce amorphous calcium carbonate for various purposes [11,12]. Brachiopods within the subphylum Linguliformea are unusual in using crystalline carbonate-substituted fluoroapatite (similar to the geological mineral francolite) in their shells [13]. Superficially resembling bivalve molluscs, the 300 or so living brachiopod species, which include the linguliform genera *Lingula* and *Discinisca* (order Lingulida, families Lingulidae and Discinidae, respectively), are a small vestige of an ancient phylum of marine invertebrates with known fossil representatives dating back as far as the early Cambrian period [14,15]. To assess whether the GAG–mineral interaction which is so widespread in vertebrate hard tissue is found also among invertebrates, we examined the shell material from two phosphatic brachiopod species, *Lingula anatina* and *Discinisca tenuis*, by SSNMR. (A preliminary account of this work has been presented as a conference abstract [16].)

2. MATERIAL AND METHODS

Shells of *L. anatina* and *D. tenuis* from animals collected off the coasts of Hong Kong (by William Chung) and Namibia (by the late Sir Alwyn Williams), respectively, were obtained (by M.C.) for the purpose of this study. The material had been preserved in ethanol. We were able to examine shell material from a recently collected *L. anatina*, which had been stored in ethanol for only a few weeks; its SSNMR properties resembled in all respects those of *L. anatina* shell material, which had been stored in ethanol for much longer periods. In our experience, prolonged exposure of bone to ethanol does not alter its SSNMR or X-ray diffraction properties. We have therefore assumed that the SSNMR properties of materials from both species are not sensitive to ethanol storage, and that differences observed between the materials from both species are

not attributable to differences in storage conditions. Furthermore, we have examined numerous samples of connective tissue, including bone and cartilage, from the same anatomical location from many individuals and found insignificant inter individual variation in their SSNMR spectral properties. We believe that inter individual variation within a given brachiopod species will also be correspondingly insignificant.

The bone sample was from ribs of a fresh trout sourced from a local fishmonger; it was stored at -20°C when not actually undergoing NMR analysis. Pure chitin from crab carapace and synthetic crystalline hydroxyapatite were purchased from Sigma Chemical Co. (Poole, Dorset, UK), and Sigma–Aldrich Laboratory Chemicals (Seelze, Germany), respectively. A specimen of geological francolite (museum number GLAHM 111221) was obtained from the Hunterian Museum, University of Glasgow. Samples were prepared for NMR and X-ray powder diffraction (XRPD) by powdering in a laboratory ball mill (Sartorius Micro Dismembrator S, 1 min at 3000 rpm) at liquid nitrogen temperature.

2.1. Solid-state nuclear magnetic resonance spectroscopy

All experiments were performed using standard SSNMR methodology on a Bruker 9.4 tesla Avance-400 spectrometer with a triple resonance probe, at frequencies of 400.1 MHz (^1H), 161.9 MHz (^{31}P) and 100.5 MHz (^{13}C) and at an MAS rate of 12.5 kHz, with samples packed into 4 mm zirconia rotors. All samples were first characterized using standard cross-polarization (CP) MAS techniques (^1H $\pi/2$ pulse length 2.5 μs , ^1H CP field 70 kHz, ^1H – ^{31}P CP contact time 10 ms, ^1H – ^{13}C CP contact time 2.5 ms, broadband TPPM decoupling during signal acquisition at a ^1H field strength of 100 kHz). $^{13}\text{C}\{^{31}\text{P}\}$ REDOR measurements were performed by applying a series of rotor-synchronized ^{31}P π pulses (8 μs) separated by 80 μs subsequent to ^1H – ^{13}C CP, with a ^{13}C refocusing π pulse (8 μs) at the midpoint of the ^{31}P pulse train. The number of rotor cycles of the REDOR sequence was varied for the purpose of exploring the effects of different dephasing times. Repetition time was 2 s.

2.2. X-ray powder diffraction

This was performed on a Philips X'Pert Pro powder diffractometer equipped with an X'celerator RTMS detector, using Ni-filtered $\text{CuK}\alpha$ radiation. Data collection was performed in a 5 – 80° range using samples on a flat plate, with a scanning step size of 0.008° , time per step of 10.8 s and scan speed of $0.0985^{\circ}\text{ s}^{-1}$.

3. RESULTS

3.1. The organic matrix

The ^{13}C SSNMR spectra of *L. anatina* and *D. tenuis* shells (figure 1) show prominent signals from chitin [17], a homopolymer of β -(1–4) glycosidically linked 2-deoxy-2-*N*-acetylaminoglucose sugars, assignments of which following Kono [18] are shown in figure 1,

although it is possible that other structurally similar aminosugars such as *N*-acetylgalactosamine contribute to these signals. There are other signals consistent with proteins; wherever possible these are assigned in figure 1 to specific amino acid residues or types, or the more prominent are asterisked where this is not possible. Except for chitin, the spectra of the two brachiopod materials are strikingly different, which may be readily appreciated by comparing the distribution and relative intensities of the prominent protein signals.

3.2. The mineral phase

Figure 2 compares the XRPD properties of *L. anatina* and *D. tenuis* shells, bone, geological francolite and pure hydroxyapatite. The last two pure materials show sharp reflections at very similar 2θ values, reflecting their crystallographic similarity. Bone reproduces the stronger reflections of hydroxyapatite, greatly broadened however on account of the poorly crystalline and nanoparticulate nature of bone mineral [19]. The *L. anatina* [20] and *D. tenuis* shell materials show XRPD patterns that replicate the reflections of francolite. The sharpness of the reflections from the shell materials shows that their mineral is much more crystalline than that of bone.

3.3. The organic matrix–mineral interface

Results of $^{13}\text{C}\{^{31}\text{P}\}$ REDOR experiments on brachiopod (*L. anatina*) shell material are exemplified in figure 3 and compared with results obtained on trout bone. In bone it is a signal at *ca* 76 ppm and another at high frequency at *ca* 182 ppm consistent with carboxylate carbons which respond most significantly when the magnetic dipole–dipole through space ^{31}P – ^{13}C interaction between the mineral and the matrix is reintroduced by the REDOR procedure. The 76 ppm signal is attributable to polysaccharide ring carbons [4,21], and the 182 ppm signal to carboxylate groups in acidic GAG polysaccharides and proteins. There are effects on other signals that are from collagen and possibly other less abundant proteins, and from mineral carbonate which gives rise to the shoulder at a chemical shift of about 168 ppm. In striking contrast, there is no dephasing of any signals in the spectra of either brachiopod shell material even at the longest practical ^{31}P – ^{13}C recoupling times.

4. DISCUSSION

The SSNMR characteristics of healthy vertebrate bone are strongly conserved between anatomical location, species and age group. Thus, the ^{13}C spectrum and $^{13}\text{C}\{^{31}\text{P}\}$ REDOR responses of trout bone are virtually indistinguishable from those of mammalian bone [4,8]. In contrast, the spectra of the two brachiopod materials are strikingly different from each other (except for the chitin components). Most of the differences relate to protein signals, which supports previous work demonstrating differing amino acid composition of the shells [22,23]. Unfortunately, in complex materials such as these it is very difficult to understand more about the

Figure 1. ^{13}C solid-state NMR spectra of the powdered shells of *L. anatina* (a) and *D. tenuis* (b) and of pure chitin (c). The structural formula is that of the repeating monomer unit of chitin; chitin signals are connected across the three spectra by dashed vertical lines. Other signals are probably ascribable to proteins, and some of the more prominent are asterisked or assigned to specific chemical functional groups where possible (R—arginine). The vertical scale of each spectrum is arbitrary but relative intensities of signals within a given spectrum are, to a reasonable approximation, proportional to the abundance of the functional group giving rise to that signal.

protein constituents beyond the fact that they must differ significantly. Nevertheless, SSNMR provides a rapid spectral 'fingerprint' by which qualitative differences between shell materials can be ascertained non-destructively. On the other hand, the fluoroapatitic

mineral phases of each shell give XRPD patterns, and ^{31}P SSNMR spectra (data not shown), which are very similar to each other. This suggests that differences in the organic matrix, at least as reflected by these techniques, do not translate into significantly different mineral

Figure 2. X-ray powder diffractograms from geological francolite (a), *D. tenuis* shell (b), *L. anatina* shell (c), bone from an aquatic vertebrate (trout) (d) and synthetic commercial hydroxyapatite (e). 2θ = X-ray diffraction angle in degrees; I = X-ray reflection intensity in arbitrary units.

phases, suggesting little influence of the matrix over mineral formation and properties at the molecular level. This is strengthened by the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR data.

Why are the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR SSNMR characteristics of the two brachiopod shell materials so different from those of bone? Firstly, it is important to recall that the REDOR phenomenon operates only between atoms within *ca* 1 nm of each other, so seeing a REDOR effect implies that the atoms concerned are either close to each other in the same molecular structure or, in different molecular structures, connected by strong physico-chemical interactions, such as electrostatic forces or hydrogen bonding. In bone, associations of this type between mineral and organic matrix are strongly evident. However, any such association in brachiopod shell biomineral is too insignificant to be observed by this technique.

The organophosphatic shells of brachiopods consist of regular microscopic-scale alternations of mineralized and organic layers [24,25]. Glucosamine sugars and acidic proteins are reported to be prominent in the biomineral layers, with galactosamine sugars abundant in the organic layer only [26]. We have proposed that the close relationship between GAG and mineral in vertebrate calcium phosphates argues for a role of the GAGs in the mineralization process [4,5]. This role may include controlling the ionic environment and initial solidification, modulating and directing calcium phosphate precipitation, and helping to prevent the inappropriate propagation of highly crystalline mineral, which might otherwise lead to structural weakness. In phosphatic brachiopod shell, this interaction is not

operating, or at least is not significant enough for its consequences to be detectable by NMR, and the XRPD shows evidence that the initiation and propagation of long-range orderly crystallization, which the GAGs may be inhibiting in vertebrates, has occurred.

However, this is not to say that there is no control over mineralization in brachiopod shells. It is likely that components of the organic matrix direct the deposition of mineral at a microscopic level, in much the same way as collagen directs the deposition and disposition of mineral particles in nascent vertebrate bone so that mineral assumes an approximate complementarity to the pre-existing organic matrix. However, neither the polysaccharides nor the proteins observable in the brachiopod shells are apparently closely composited with the fluoroapatite mineral at the atomic level. Brachiopod polysaccharides may not be of appropriate composition to influence mineralization in the same way as vertebrate GAGs, which are rich in anionic sulphated and carboxylic acid-containing sugar derivatives. Possibly, the structure of the chitinous matrix supports a relationship between organic scaffold and solidifying mineral, which predisposes to high crystallinity. There may be proteins in linguliform shell material that actively promote the formation of crystalline apatite, just as they appear to promote the conversion of amorphous calcium phosphate into apatite [27]. It must be remembered that the brachiopod mineral is chemically different from that of vertebrate mineral (fluoroapatite-like versus hydroxyapatite-like) and some of the differences in crystallinity may also be due to different physico-chemical properties of the mineral phases.

Lingula has a crack-resistant and relatively flexible shell, of some advantage to an animal which spends its life buried in sediment, whereas discinids such as *Discinisca* live on top of hard substrates, to which they are attached by means of a muscular pedicle [28]. It is therefore likely that the structures of the mineral-organic matrix in *Lingula* shell and *Discinisca* shell have been naturally selected to fulfil different roles and, by comparison, provide clues to how structure and composition relate to differing biomaterial properties. Differences in inter- and intracrystalline organic matrix organization have implications for properties such as hardness, flexibility and the ability to resist crack propagation [29]. For example, the increased flexibility and reduced brittleness of the *Lingula* compared with other brachiopod shells is ascribed to the presence of more distinctive and thinner laminated layers of interleaved fibrous organic, and crystalline, matter [30]. It may be that this and similar mechanisms are preferable under some circumstances to employing atomic-level associations with GAG and protein to achieve a similar end. An ordered, thermodynamically stable crystalline material which is more reliant on anatomical organization than molecular interactions with organic matrix may be more robust in extremes of moisture, ambient osmotic potential and temperature.

In summary, the contrast observed between vertebrate bone, with powerful atomic-scale GAG-mineral and protein-mineral interactions and crystallographically poorly ordered mineral, and the brachiopod shells,

Figure 3. $^{13}\text{C}\{^{31}\text{P}\}$ REDOR data from *L. anatina* (a) shell, and trout bone (b). Each experiment is performed in two phases. First a reference spectrum (black trace) is acquired in which the through-space interatomic interaction between ^{13}C and ^{31}P nuclear magnetic dipoles is suppressed by the magic angle sample spinning procedure. Then a REDOR spectrum (grey trace) is acquired while this interaction is restored by the application of a train of ^{31}P π pulses applied in synchrony with the magic angle spinning period. The practical effect of this is to 'dephase' signals from ^{13}C atoms that are close in space to ^{31}P atoms, which manifests as a reduction in intensity of the relevant ^{13}C signal. Usually the experiment is repeated with increasing dephasing times, which enables carbons at increasingly large separations, effectively up to about 1 nm, from phosphorus to be identified. The data shown were acquired with a (relatively long) 9.6 ms dephasing time. In the bone spectra, the GAG ring (76 ppm) and carboxylate (182 ppm) signals, which undergo strong dephasing, are arrowed, as are some of the prominently affected matrix protein signals. Spectra of *D. tenuis* shell material which, like that of *L. anatina*, are devoid of REDOR effects, are not shown.

with undetectable atomic-scale polysaccharide–mineral and protein–mineral interactions, and crystallographically well-ordered mineral, may be further evidence for the central role of GAGs and other macromolecules in directing solidification of mineral in vertebrates into bi-materials that are simultaneously hard (resisting shape change) and tough (requiring great strain before breaking). On the other hand, the absence of atomic-scale GAG–mineral interaction in brachiopods is accompanied by high mineral crystallinity, which is undesirable in bone because it predisposes to brittleness. Natural selection of GAG participation in vertebrate mineralization, and the resultant material properties of their hard tissues, may have been a significant step in vertebrate evolution. The brachiopods appear to have evolved a different means of achieving similar ends with their hard tissue, perhaps based on microscopic-scale laminae. Brachiopod materials have clearly responded to very different evolutionary pressures from vertebrate endoskeletal bone, and their structural composition must be considered in this light.

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