Mineral Surface in Calcified Plaque Is Like That of Bone
Further Evidence for Regulated Mineralization

Melinda J. Duer, Tomislav Friščić, Diane Proudfoot, David G. Reid, Michael Schoppet, Catherine M. Shanahan, Jeremy N. Skepper, Erica R. Wise

Objectives—Cell biological studies demonstrate remarkable similarities between mineralization processes in bone and vasculature, but knowledge of the components acting to initiate mineralization in atherosclerosis is limited. The molecular level microenvironment at the organic–inorganic interface holds a record of the mechanisms controlling mineral nucleation. This study was undertaken to compare the poorly understood interface in mineralized plaque with that of bone, which is considerably better characterized.

Methods and Results—Solid state nuclear magnetic resonance (SSNMR) spectroscopy provides powerful tools for studying the organic–inorganic interface in calcium phosphate biominerals. The rotational echo double resonance (REDOR) technique, applied to calcified human plaque, shows that this interface predominantly comprises sugars, most likely glycosaminoglycans (GAGs). In this respect, and in the pattern of secondary effects seen to protein (mainly collagen), calcified plaque strongly resembles bone.

Conclusion—The similarity between biomineral formed under highly controlled (bone) and pathological (plaque) conditions suggests that the control mechanisms are more similar than previously thought, and may be adaptive. It is strong further evidence for regulation of plaque mineralization by osteo/chondrocytic vascular smooth muscle cells. (Arterioscler Thromb Vasc Biol. 2008;28:2030-2034)

Key Words: atherosclerosis ■ biomineralization ■ glycosaminoglycans ■ nuclear magnetic resonance spectroscopy ■ vascular calcification

Calcification is prevalent in numerous vascular pathologies and is associated with adverse clinical outcomes. Parallels have frequently been drawn between ectopic calcification processes in vessels and normal developmental osteogenesis. In fact vascular calcified material is by many criteria indistinguishable from bone, and its formation involves the participation of many cellular and molecular signaling processes underlying normal osteogenesis. These include matrix vesicle release, expression of mineralization-regulating proteins by vascular smooth muscle cells (VSMCs) of the vessel wall, and the association of calcification with lipid and chronic inflammation, a hallmark of atherosclerosis. Indeed vascular calcification is recognized as regulated biomineralization instead of merely passive precipitation. However questions are still unanswered concerning the mechanisms leading to the initiation of calcification in the vessel wall particularly as it occurs in different vascular environments (ie, media and intima), which do not mimic that of bone. Moreover, VSMCs can overexpress mineralization inhibitors such as matrix Gla protein (MGP) to effectively block mineralization.

Aspects of the molecular structure of mineralized tissues can be studied by the solid state NMR (SSNMR) technique $^{13}$C[$^{31}$P] rotational echo double resonance (REDOR). $^{13}$C is a stable, nonradioactive, NMR-receptive isotope of carbon occurring naturally at about 1.1% abundance; similarly $^{31}$P is a stable NMR-receptive isotope of phosphorus which however is naturally present at 100% abundance. A $^{31}$C NMR spectrum records a signal for each chemically distinct carbon site in the sample. In a REDOR experiment a $^{13}$C NMR spectrum is recorded with and without a train of radiofrequency pulses applied to the $^{31}$P spins before acquisition of the spectrum, forming the so-called nondephased and dephased $^{13}$C spectra, respectively. The nondephased $^{13}$C spectrum recorded acts as a reference, whereas in the spectrum recorded with the $^{31}$P pulse train, signals attributable to $^{13}$C spins in close proximity to $^{31}$P spins in the material will show a reduction in intensity. Thus, those $^{13}$C sites which are spatially close to $^{31}$P are immediately identified from the resonance frequency of those signals showing reduction in intensity. By following the reduction in intensity of a given $^{13}$C signal as a function of the time for which the $^{31}$P pulse
train is applied, quantitative determination of the $^{13}$C-$^{31}$P distance is possible. The REDOR effect is only transmitted over interatomic distances of less than 10 Ångstroms (1 nm), so it is a conclusive proof of a true molecular composite material in which individual components are bound in associations mediated by intermolecular interatomic forces (for instance electrostatic and hydrogen bonding) and not merely mixed together. The interface between the organic matrix and calcium phosphate biomineral particles in plaques and bone is particularly amenable, because practically all $^{13}$C is in the former and all $^{31}$P in the latter. This makes the experiment highly selective for the interface itself. In bone it shows that the molecular constituents of the organic-mineral interface are rich in polysaccharides, most likely glycosaminoglycan (GAG) sugars, rather than proteins. Therefore, a comparative analysis of this interface may provide clues to the exact nature of similarities and differences between mineralization processes in the vasculature, and bone, that cannot be revealed by cell biological studies alone.

Materials and Methods

Calcified Atherosclerotic Plaque

Material was collected with appropriate ethical approval during carotid endarterectomy procedures. The human tissue (n=4) was put into sterile flasks with 15 mL medium (M199 with penicillin and streptomycin) and 1 mL elastase (from a stock solution of 5 mg/mL porcine pancreatic Type IV elastase, Sigma) and 5 mL collagenase (from a stock solution of 9 mg/mL C. hemolyticum Type I collagenase, Sigma) added, shaken for 6 hours in a waterbath at 37°C, centrifuged at 800 r.p.m. for 5 minutes, washed with cold sterile phosphate buffered saline, spun again, and the resultant pellet frozen and stored for analysis. The procedure is necessary as SSNMR is a relatively insensitive technique, so it is highly desirable to remove the sample from the sample as much extraneous material as possible in order to concentrate the sites of interest. To determine whether this pretreatment of the material interfered with the SSNMR measurements, it was compared with untreated tissue that was frozen before grinding. For all SSNMR measurements, thawed material was lightly ground with a pestle and mortar and packed into 4-mm outer diameter zirconia magic angle spinning (MAS) rotors (Bruker).

A bone from an adult horse used for general purpose exercise and euthanized for humanitarian reasons unconnected with this study was used for comparison. Several bone specimens obtained at surgery were found to be identical to those obtained at autopsy, indicating that the difference in harvesting between plaque (surgery) and bone (autopsy) does not introduce noticeable differences. In the cases of both mineralized plaque and bone, the grinding produced particles the order of several tens of micrometers in size; as typical biomineral particles are much smaller than this and the interatomic interactions we are exploring operate over subnanometer length scales, we believe the grinding will not affect our observations of these interactions significantly, if at all. In previous studies on bone, we have explored a variety of ways of preparing the sample material, including slicing/chopping and grating, as well as grinding to different particle sizes and saw no difference in spectra between different preparation methods.

NMR Spectroscopy

This was performed on a Bruker Avance-400 triple channel spectrometer at a polarizing magnetic field strength of 9.4 T (Tesla), with samples packed into 4-mm outer diameter zirconia rotors. These enable the samples to be spun rapidly (at a rotational frequency of 7 kHz in these experiments) at an angle of 54.7° to the polarizing magnetic field, the so called “magic angle.” This “magic angle spinning” (MAS) is necessary to remove certain magnetic interactions between the NMR active nuclei in the sample which would otherwise broaden the resonances and result in featureless spectra. Where there was insufficient material to fill the rotor, the dead space was packed out with Teflon tape which gives no NMR signal with the data acquisition methods we have used. The following spectral acquisition parameters were used: Resonance frequencies: $^1$H 400.4 MHz, $^{13}$C 100.7 MHz, $^{31}$P 162.1 MHz; $^1$H π/2 pulse 2.5 μs, mean ramped cross polarization (CP) field strength 70 kHz, CP time 2.5 ms, $^3$P decoupling field 100 kHz, recycle delay 2 s, MAS rate 12.5 kHz. For the $^{31}$P spectra 128 signal accumulations were averaged for satisfactory signal to noise; $^{13}$C spectra required averaging of between 20 and 40 k signal accumulations. This difference reflects the 100% and 1.1% abundance of the $^{31}$P and $^{13}$C isotopes, respectively. $^{13}$C($^{31}$P) REDOR was performed with $^1$H-$^{13}$C CP, $^{31}$P π pulses of 8.4 μs, the separation between the center of the $^{31}$P pulses being 80 μs and synchronous with the MAS, and a $^{13}$C refocusing π pulse of 10 μs inserted at the middle of the recoupling $^{31}$P pulse train. By convention, the notation $^{13}$C($^{31}$P)" signifies that it is the signal from $^{31}$C magnetic nuclear spins which is observed directly (at 100.7 MHz), whereas the $^{31}$P magnetic nuclear spins are caused to interact with them by a suitable combination of nuclear spin energy level excitations applied at 162.1 MHz.

X-Ray Powder Diffraction (XRPD)

This was performed on a Philips X’Pert Pro powder diffractometer equipped with an X’celerator RTMS detector, using Ni-filtered CuKα radiation. Data collection was performed in a 5 to 80° range using samples on a flat plate, with a scanning step size of 0.008°, time per step of 10.8 seconds and scan speed of 0.0985%/s.

Scanning Electron Microscopy (SEM)

Extracted mineral was dusted onto spectroscopically pure double-sided carbon tabs mounted on Cambridge SEM stubs. It was viewed in a FEI Phillips XL30 FEGSEM operated at 5kv.
Results

Figure 1 compares typical $^{13}$C SSNMR spectra of calcified plaque, and bone; supplemental Figure I (available online at http://atvb.ahajournals.org) shows data from all samples and clearly shows a high degree of reproducibility. Collagen accounts for most signals. Notable exceptions are a broad signal at about 103 ppm, weak but present in all samples, and another at 76 ppm, which tends to be more prominent in mineralized plaque than in bone. These are consistent, respectively, with the anomeric carbons, and some of the other pyranose 6-membered sugar ring carbons, of $\beta$-linked polysaccharides like GAGs. To highlight the relationship between GAG signals and GAG chemical structure, a chemical representation of the repeat subunit (-glucuronic acid $\beta$ (1–3) N-acetylgalactosamine-4-sulfate $\beta$ (1–4)-) of the widely occurring GAG chondroitin-4-sulfate is included. The signal attributable to most of the GAG sugar ring carbon atoms, the signal attributable to the GAG sugar anomeric carbons (marked * in the chemical structural formula and the spectra), and the signal attributable to the GAG acidic carboxylate carbons (marked # in the chemical structural formula and spectra) are identified. The latter also contains signal from protein acidic carboxylate carbons; it presents a prominent shoulder on the high frequency edge of the signal envelope centred at about 175 ppm, itself attributable to overlap of numerous protein (and GAG) carbonyl signals. Spectra of treated and untreated human carotid endarterectomy material are shown in supplemental Figure II. This clearly shows that collagenase/elastase pretreatment did not affect the GAG composition or structure in the material.

Figure 2 shows X-Ray powder diffraction (XRPD) and $^{31}$P SSNMR data proving the mineral is essentially hydroxyapatite- and bone-like. However the XRPD trace of microcrystalline hydroxyapatite contains numerous sharp reflections consistent with a highly ordered crystalline environment; in consequence of this order the $^{31}$P NMR spectrum is also narrow, showing that all phosphorus atoms experience very similar environments. The XRPD trace and $^{31}$P spectrum of bone show much broader features consistent with less perfectly crystalline mineral, and greater environmental heterogeneity attributable to molecular disorder and mineral surface effects. The XRPD trace and $^{31}$P NMR spectrum of calcified plaque is intermediate between these extremes, probably reflecting greater mineral crystallinity and larger average mineral crystallite size. This is borne out by SEM illustrating the typical heterogeneous micro/nanocrystalline nature of the calcifications.

Figure 3 presents typical $^{13}$C($^{31}$P) REDOR results reflecting the mineral-organic interface composition; more comprehensive data on other samples is presented in supplemental Figure III. The black trace is the reference spectrum, which in this case is largely due to the various components of collagen. As described in the Introduction, this spectrum contains signals from all $^{13}$C sites in the sample. The dephased spectrum in red is obtained after the application of radiofrequency pulses to the $^{31}$P nuclei in the sample. As already explained, the effect of these pulses is to reduce in intensity any signal due to a $^{13}$C nucleus close in space to a $^{31}$P; close in space in this context is less than 10 Å. As in bone, the most affected signals, which can therefore be associated with molecules in intimate association with mineral, are the 76 ppm polysaccharide peak, and signals at 175 and 181 ppm which are probably from amide carbonyl, and carboxylate, groups, which are abundant in GAG polysaccharides (as well
NMR spectra of mineralized plaque at 2 different \(^{31}\text{P}\)-\(^{13}\text{C}\) dipolar recoupling times. Inset: adjacent histology sections from mineralized plaque, one stained for polyanions with alcian blue, the other for polyanions with alcian blue and calcification with von Kossa.

**Figure 3.** \(^{13}\text{C}(^{31}\text{P})\) rotational echo double resonance (REDOR) NMR spectra of mineralized plaque at 2 different \(^{31}\text{P}\)-\(^{13}\text{C}\) dipolar recoupling times. Inset: adjacent histology sections from mineralized plaque, one stained for polyanions with alcian blue, the other for polyanions with alcian blue and calcification with von Kossa.

as in some proteins, of course). The intensity reduction should increase with increasing time for which the radiofrequency pulses are applied to the \(^{31}\text{P}\) nuclei; this is illustrated in supplemental Figure IV, and compared with that of bone in supplemental Figure V. Also in common with bone, other signals suffer a smaller amount of intensity loss; some of these are from collagen but others may be from exocyclic 6-carbons (ca. 65 to 70 ppm), aminated or amidated ring carbons (ca. 54 ppm), or N-acetyl methyl groups (ca. 25 ppm) of GAGs and other polysaccharides. Thus, the macromolecules most strongly associated with the mineral phase in calcified plaque are polysaccharides, probably GAGs. We have also examined samples histologically after staining with von Kossa stain, specific for calcified deposits, and alcian blue, which recognizes polyanions; the Inset shows typical data and more appears in the supplemental material. We find that calcification (dark brown) always occurs in alcian blue staining regions which are probably GAG-rich (blue), although not all alcian blue staining regions calcify. Moreover, GAG-rich calcified regions were often found surrounding lipid pools—areas previously described as colocalizing with mineralization.\(^{11}\) This suggests that calcification tends to occur in polyanion-rich regions, where the polyanions are most likely GAGs as these are the predominant polyanions present in these tissues. Coincidence of GAG and calcification at the micron resolution length scale of light microscopy is not synonymous with the subnanometer atomic length scale intermolecular bonding revealed by the NMR, of course, though it is consistent with the hypothesis this article presents.

**Discussion**

We have shown that the matrix-mineral atomic interface in calcified atherosclerotic plaque is very similar to that in bone and, surprisingly, is characterized by a predominance of GAGs. Colocalization of GAGs and mineralization has been detected histologically in calcifying tissue,\(^{12-14}\) and importantly there is accumulating evidence that the type of GAG in the matrix may exert tight control over mineralization, particularly during its initiation.\(^{15,16}\) An early event in bone mineralization is release of membrane-bound matrix vesicles which act as the nidus for mineral nucleation by concentrating calcium and phosphate. Matrix vesicles bind GAGs, and this binding is increased in mineralization-competent hypertrophic chondrocyte-derived matrix vesicles, further suggesting that GAGs play a direct role in initiating physiological mineralization.\(^{17}\) However, as mineralization progresses, GAGs may also act to prevent uncontrolled propagation of calcium phosphate crystallization. Further evidence for this comes from human disease where dysregulated expression of GAGs leads to bone defects, whereas a lower incidence of plaque calcification in chronic asthma sufferers, who show elevated levels of circulating GAGs like heparin, has been reported.\(^{18}\)

Atherosclerotic lesions contain proteoglycan, intermixed with loosely scattered collagen fibrils,\(^{19}\) as plaques develop secretion and accumulation of GAGs occurs within the extracellular matrix. Also, GAGs bind low-density lipoprotein,\(^{20}\) retaining cholesterol in the plaque and enhancing local proinflammatory signals. Lipid and mineralization are coincident in plaques,\(^{4,11}\) and inflammatory signals at lipid accumulation sites initiate calcification by inducing vascular smooth muscle cell (VSMC) death and vesicle release.\(^{21-24}\) Vesicles contain hydroxyapatitic nanocrystals representing the initial calcification seed. Thereafter, VSMCs that have undergone osteo/chondrocytic conversion are found abutting the microlcalcifications.\(^{21}\) Taken together with this study, showing the remarkable similarities between the resultant biomineral found in plaques, and bone, it seems reasonable to postulate that GAG binding to lipid may initiate VSMC injury and death. This leads to an initial calcification nidus, followed by osteogenic phenotypic conversion of VSMCs, although the mineralization process may also trigger GAG production.

Indeed, the similarities between bone and carotid arterial mineralization which we show support the contention that this may be protective, by rapidly dampening unregulated deposition of mineral initiated by injury. Part of this response may be to produce biominal that is inert, rather than proinflammatory such as in synovial fluid.\(^{25}\) It is plausible that this is a common feature of all vascular calcification but this requires more evidence to establish.

As in bone, GAGs have attracted less attention in plaque than proteins,\(^{26}\) such as matrix GlA protein and osteocalcin,\(^{27}\) decorin,\(^{28}\) osteopontin,\(^{29}\) bone sialoprotein,\(^{4}\) and collagen itself,\(^{30}\) which have all been proposed as important mineral-
ization modulators. Nonetheless, many of these are often heavily glycosylated, and it may be part of their role to target GAGs to specific sites in developing, and mineralizing, matrices. However, it is particularly interesting that these proteins were not more abundant in atherosclerotic mineral and that other vascular specific extracellular matrix proteins such as elastin, often postulated to be a nucleator of hydroxapatite in the vessel wall, were not present in close association with mineral. This further supports the notion that some vessel wall biomineralization may be adaptive, and this has implications for its treatment.

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Disclosures

None.

References

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Figure I. **Intersample variability.** $^{13}$C solid state NMR spectra of the four samples studied, all shown after collagenase and elastase digestion. The similarity between samples is not as marked as what we observe for tissue which mineralizes normally, like bone. Although other macromolecules, and lipid in the case of sample D, probably contribute, the signals characteristic of collagen and GAGs are strongly conserved (cf. Figure 1 of the main paper). In particular the “signature” signals of the GAG sugar ring carbons, and of the $\gamma$-carbon of hydroxyproline, an amino acid which is distinctive of collagen, are highlighted by red and black arrows respectively in each dataset.
Figure II. Effects of collagenase/elastase digestion. Comparison between $^{13}$C solid state NMR spectra of calcified plaque (designated sample B in remaining online material) before (bottom) and after (top) collagenase and elastase digestion. Not surprisingly the procedure does change the overall appearance of the spectrum, in particular several sharp signals become much less prominent or disappear. These are probably due to metabolites which are more mobile (on a molecular motional scale) than the extracellular matrix structural macromolecules, most likely lipids. However all the prominent features ascribable to GAG and collagen protein are retained. Some of these are indicated and assignments, corresponding to those in Figure 1 of the main paper, are shown.

Another factor in the decision to study digested material is dictated by the practicalities of the solid state NMR experiment. This involves spinning the sample rapidly in a small rotor at a rotation frequency (for our experiments) of 12.5 kHz. This places great demands on rotor stability, and sample homogeneity is paramount in ensuring this. Before digestion samples are heterogeneous, with calcified bodies intermixed with lipid rich organic material, which is not conducive to efficient sample spinning, particularly for the REDOR experiments which require control of the spinning frequency to within +/- 1 Hz. We believe however that the material removed by the brief digestion we used is incidental to mineralization, and that the organic material left behind is that which is truly compositied with mineral and therefore the focus of this study.
Figure III. $^{13}\text{C}^{[31}\text{P}]$ REDOR intersample variability. A comparison of the REDOR effects seen for three of the samples at a single phosphorus-carbon magnetic coupling time of 5.6 ms. The interpretation of each pair of spectra is explained in the legend to Figure 3 in the main paper; the REDOR effect is conveyed by the difference between the black reference trace (without phosphorus-carbon magnetic coupling) and the red trace (with phosphorus-carbon magnetic coupling). In all cases there are significant effects to GAG, as well as to some protein signals. As NMR is an insensitive technique acquiring satisfactory REDOR data is time consuming, especially when the volume of sample is limited as it usually is for clinical tissue. Data at a single magnetic coupling time point can take several days to acquire and, while desirable, it is often impractical to collect data at several time points.

Quantification In simpler samples, such as pure crystalline organic compounds, it is customary to quantify the REDOR effect by integrating the intensity of each signal in the reference and the corresponding REDOR spectrum. The ratio of signal intensity in the REDOR spectrum to signal intensity in the reference spectrum is then plotted against the coupling time. This time dependence is proportional to the inverse cube power of the distance between coupled atoms, and becomes negligible for atoms more than about 1 nm apart. In favourable cases, usually where each atom is coupling with only a single other
magnetically active atom, the so-called two spin case, absolute values of the interatomic distances can be extracted, and a detailed structure for the molecule calculated.

We have not quantified our REDOR effects for two reasons. Firstly, because of signal overlap meaningful quantitation is difficult. Secondly, because of the 100% natural abundance of the magnetically active $^{31}$P isotope of phosphorus and its high density in the biomineral, each carbon atom will be coupled to at least two, probably several, phosphorus sites, presenting a situation difficult or impossible to analyze. Moreover each biopolymer will adopt a variety of (perhaps similar) conformations and spatial relationships with the mineral surface, so extracting any better than approximate information representative of an ensemble-average structure cannot be done. However there is relatively more phosphorus-carbon coupling to protein signals than we observe at similar coupling times in bone (see Fig. V), probably reflecting a less organised organic matrix in the mineralized plaque.
Figure IV. Mineralized plaque: Dependence of $^{13}\text{C}^{31}\text{P}$ REDOR on phosphorus-carbon magnetic coupling time. The traces show REDOR data acquired with four different phosphorus-carbon magnetic coupling times. Note that it is the GAG signal at 76 ppm which begins losing intensity at the shortest magnetic coupling times along with the high frequency carboxylate shoulder which also contains a GAG contribution. As we increase the magnetic coupling time the REDOR demagnetization is driven further out from the mineral surface, and its effects become more generalized and embrace numerous collagen signals. Also, the GAG signals are the only ones to decrease to baseline at the longest coupling times showing that all detectable GAG is close to, that is within about 1 nm of, the mineral surface. The bulk of the protein is further away.
**Figure V. Bone: Dependence of $^{13}$C-$^{31}$P REDOR on phosphorus-carbon magnetic coupling time.** The traces show REDOR data acquired under the same phosphorus-carbon magnetic coupling times as Figure IV. Note the same initial response of the GAG signals, and their ultimate loss of intensity down to baseline. Also note that effects to collagen are less widespread than in calcified plaque, possibly reflecting a more ordered structure with average mineral-collagen separations greater than those of plaque.

In the course of a major project we have studied a large number of bone samples, harvested at autopsy and at surgery, from horses, humans, rats, and mice. Samples have been powdered for solid state NMR by grinding in a mechanical ball mill, or by filing using a metal file. When examined under identical NMR experimental conditions there are no significant differences between any material or preparation method. Both treatments produce particles which are many microns in size, whereas the mineral particles in bone are sub-micron. Thus each powder particle contains numerous mineral nanoparticles in what must be an essentially native state. By this token we also believe that the pre-treatments we used on the plaque samples – digestion, centrifugation, and grinding in a pestle and mortar (under much lower energy conditions than we have to use to prepare bone samples) – will not have disturbed the atomic level structures and relationships we are interested in.
Blue staining is proteoglycans/GAG’s and brown staining is calcification. There is a lot of GAG staining throughout the vessel wall particularly in association with vascular smooth muscle cells (VSMCs). In general calcification occurs in GAG-rich areas but not all GAG-rich areas are calcified - as in the lesion above. Calcification occurs in this vessel in the media in association with VSMCs (arrowed).

Examples of calcification in a GAG rich area just bordering on an area of the intima that is almost devoid of GAG staining (In).
Calcified remnants of lipid pools in a GAG-rich area. Note lack of brown calcification in the surrounding GAG-poor areas (arrowed).

Serial sections showing a calcified area that is rich in GAG’s (arrowed on left) surrounded by an area poor in GAG’s that is not calcified. Co-staining of this same area is shown on the right to reveal calcification.

Calcified areas very rich in GAG’s. Med, media; In, intima.