Proton Environments in Biomimetic Calcium Phosphates Formed from Mesoporous Bioactive CaO–SiO₂–P₂O₅ Glasses in Vitro: Insights from Solid-State NMR

Renny Mathew,†‡ Claudia Turdean-Ionescu,† Yang Yu,† Baltzar Stevensson,† Isabel Izquierdo-Barba,‡§ Ana García,§§ Daniel Arcos,§§ María Vallet-Regi,§§ and Mattias Eden,†‡

†Department of Materials and Environmental Chemistry, Arhenius Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden
‡Departmento de Química Inorgánica y Bioinorgánica, Facultad de Farmacia, Universidad Complutense de Madrid, Instituto de Investigación Sanitaria Hospital 12 de Octubre i+12, 28040 Madrid, Spain
§Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain

ABSTRACT: When exposed to body fluids, mesoporous bioactive glasses (MBGs) of the CaO–SiO₂–P₂O₅ system develop a bone-bonding surface layer that initially consists of amorphous calcium phosphate (ACP), which transforms into hydroxy-carbonate apatite (HCA) with a very similar composition as bone/dentin mineral. Information from various ¹H-based solid-state nuclear magnetic resonance (NMR) experiments was combined to elucidate the evolution of the proton speciations both at the MBG surface and within each ACP/HCA constituent of the biomimetic phosphate layer formed when each of three MBGs with distinct Ca, Si, and P contents was immersed in a simulated body fluid (SBF) for variable periods between 15 min and 30 days. Directly excited magic-angle-spinning (MAS) ¹H NMR spectra mainly reflect the MBG component, whose surface is rich in water and silanol (SiOH) moieties. Double-quantum—single-quantum correlation ¹H NMR experimentation at fast MAS revealed their interatomic proximities. The comparatively minor H species of each ACP and HCA component were probed selectively by heteronuclear ¹H−³¹P NMR detection. The initially prevailing ACP phase comprises H₂O and “nonapatitic” HPO₄²⁻/PO₄³⁻ groups, whereas for prolonged MBG soaking over days, a well-progressed ACP → HCA transformation was evidenced by a dominating O⁴⁻H resonance from HCA. We show that ¹H-detected ¹H → ³¹P cross-polarization NMR is markedly more sensitive than utilizing powder X-ray diffraction or ³¹P NMR for detecting the onset of HCA formation, notably so for P-bearing (M)BGs. In relation to the long-standing controversy as to whether bone mineral comprises ACP and/or forms via an ACP precursor, we discuss a recently accepted structural core−shell picture of both synthetic and biological HCA, highlighting the close relationship between the disordered surface layer and ACP.

1. INTRODUCTION

Bone is a composite material consisting of platelets of Ca-deficient hydroxy-carbonate apatite (HCA) deposited on collagen-I fibrils, forming a complex hierarchical structure that underlies its extraordinary mechanical properties.¹,² The very thin (2−10 nm) and elongated HCA platelets (30−50 nm long; 15−30 nm wide) pack themselves like a deck of cards, with the longest crystal axis aligned with the direction of the collagen fibril.¹−⁴ Besides minor amounts of noncollagenous proteins and glycosaminoglycans, bone comprises significant amounts of citrate (∼1 wt %) and water (∼10 wt %),⁵ the latter associated with both the organic and inorganic components. While it is recognized that the H₂O content of bone is reduced on its aging, which correlates with a loss of strength and stiffness, recent research suggests that the water and citrate components may have stronger bearings on the stability and mechanical properties of bone than was previously conceived, with their role of interfacing the stacked HCA platelets by acting like a glue that enhances the tissue strength.³−⁵

The OH content of bone mineral is significantly lower than for stoichiometric calcium hydroxy-apatite (HA; Ca₅(PO₄)₃OH), which is the mother structure of biological/synthetic HCA. The deficiency of Ca²⁺, PO₄³⁻, and OH⁻ species in biological apatite stems from various coupled ion substitutions that lead to incorporation of mainly HPO₄²⁻, CO₃²⁻, and F⁻ anions, together with cations such as Na⁺, Mg²⁺, and Sr²⁺.⁶−¹⁰ The element specificity and high signal sensitivity of magic-angle spinning (MAS) ¹H nuclear magnetic resonance (NMR) have been exploited in numerous investigations targeting the various proton (H₂O, OH⁻, and HPO₄²⁻) species of bone mineral⁶−¹⁴ and synthetic H(C)A⁶,¹⁵−²² as well as
for unraveling the interfacing role of water between the HCA platelets. Noteworthy is that the mineral-associated $^1$H and $^{31}$P environments may be probed selectively by using cross-polarization (CP) MAS NMR experimentation utilizing interatomic $^1$H–$^{31}$P distance-dependent heteronuclear dipolar interactions; this allows for rejecting all proton resonances from the organic components of bone/dentin that otherwise dominate $^1$H NMR spectra recorded directly by single pulses. CPMAS-based NMR experimentation, such as the heteronuclear correlation (HETCOR) technique, also enables the discrimination of proton environments of amorphous calcium phosphate (ACP) and crystalline H(C)A. Recently, several advanced heteronuclear NMR protocols have been applied for estimating the nanometer-scale organization and the extent of (dis)ordered domains in various heterogeneous phosphate-bearing materials, encompassing bone/dentin as well as synthetic composites.

There are several NMR reports on the $^1$H speciation in directly precipitated H(C)A and as an integrated component in various synthetic multiphase materials. However, despite a vast literature on studies of HCA growth in vitro from biomaterials intended for bone/tooth implants, $^1$H NMR studies targeting the proton environments in ACP and HCA generated biomimetically from such substrate biomaterials are very sparse. One class of amorphous biomaterials is melt-prepared silicate-based bioactive glasses (BGs) and their templated mesoporous bioactive glass (MBG) counterparts with an ordered mesopore arrangement, when subjected to simulated body fluid (SBF), biomimetic HCA forms at the (MBG) base surface via an ACP precursor, as first proposed by Hench and co-workers. In vivo, this bioinert-mimicking phosphate layer interfaces strongly with bone tissue.

A detailed insight into both the fundamental ACP/HCA formation mechanisms and the similarities/differences of the phosphate and proton speciations of biomimetic HCA and biological apatite is desirable. For three series of MBGs with variable Ca, Si, and P contents and SBF-exposure intervals ranging between 15 min and 30 days, we present a comprehensive NMR study unraveling the evolution of the proton environments both at the MBG surface and within each ACP and HCA component of the growing biomimetic phosphate layer. The silicate and phosphate environments of the same set of specimens were previously characterized by $^{29}$Si and $^{31}$P MAS NMR studies, which are summarized in Section 3. They will be contrasted with the complementary information gained herein about the H species of the heterogeneous multicomponent specimens.

We provide results from single-pulse $^1$H NMR and fast-MAS $^1$H–$^1$H double-quantum/single-quantum (2Q–1Q) correlation NMR experiments, which inform primarily about the various water and silanol (SiOH) moieties and their interatomic proximities of the dominating H reservoir at the MBG silicate surface, whereas the overall minor H populations of each ACP and HCA component of the phosphate layer were examined by heteronuclear $^1$H–$^{31}$P NMR experimentation. The present work extends our previous $^1$H NMR studies, which only discussed the surface proton speciations in detail for the pristine MBGs, whereas the counterparts in SBF-soaked specimens were restricted to one MBG base composition and a limited number of exposure periods, more involving a much higher MBG loading in the SBF (20 g/L) than the current one (0.6 g/L).

The details of how bone mineral nucleates and to what extent this process is regulated by noncollagenous proteins and collagen remain unclear. More remarkable is the longstanding controversy whether bone mineral is formed via transient precursor phases (OCP and/or octacalcium phosphate (OCP)) or directly precipitated H(C)A. Recently, several advanced heteronuclear NMR techniques, such as the heteronuclear correlation (HETCOR) technique, also enable the discrimination of proton environments of crystalline calcium phosphate (ACP) and crystalline H(C)A. The recent bone and enamel mineralization studies in vertebrates, leading to an alternative view that bone mineral crystallizes directly as tiny platelets of poorly ordered and highly ion-substituted HCA. Nonetheless, recent insight on the structural organization of both synthetic and biological apatite involves a crystalline HCA core and a structurally disordered surface layer, which is then readily rationalized, and the two seemingly disparate viewpoints may naturally be reconciled: this possibility—which generally seems to be overlooked in the literature—is commented on in Section 5.2 and will be discussed more thoroughly elsewhere (M. Edén, to be published).

2. MATERIALS AND METHODS

2.1. MBG Preparation. Three MBG specimens of nominal molar compositions 10CaO–90SiO$_2$ ("90"), 10CaO–85SiO$_2$–5P$_2$O$_5$ ("85S5"), and 37CaO–58SiO$_2$–5P$_2$O$_5$ ("85S5") were prepared by an evaporation-induced self-assembly (EISA) procedure at 40 °C, using the nonionic P123 triblock copolymer as a structure-directing agent and precursors of tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP), and Ca(NO$_3$)$_2$·4H$_2$O to incorporate Si, P, and Ca, respectively. The detailed conditions are described in ref 69. The resulting homogeneous membranes were heated at 700 °C for 6 h to remove nitrate ions and organic molecules. The textural properties and the experimentally determined cation compositions of the pristine MBGs are listed in Table 1.

2.2. SBF-Exposed MBG Specimens. An SBF solution was prepared according to Kokubo et al. by dissolving NaCl, KCl, NaHCO$_3$, K$_2$HPO$_4$·3H$_2$O, MgCl$_2$·6H$_2$O, CaCl$_2$, and Na$_2$SO$_4$ into distilled water. It was buffered at pH = 7.38 by using tris(hydroxymethyl)-aminomethane/HCl (TRIS/HCl) and then passed through 0.22 μm Millipore filters to avoid bacterial contamination. An amount of 600 mg of a fine powder (<20 μm particle diameter) of each pristine MBG was immersed in 1.00 L of SBF in a sealed polyethylene container in an Ecotron HT incubator at 37 °C. The solutions were stirred at 100 rpm for each of the following periods: 0.25, 1, 4, 8, 24 h.
and {3, 7, 15, 30} days. The solid phase was subsequently retained by filtration, soaked in acetone to quench the surface reactions, washed with water, and finally vacuum dried at 37 °C for several days. The SBF-exposed specimens are henceforth denoted S90-Q5SBF, S85-Q5SBF and S58-Q5SBF, with the immersion period tSBF specified either in hours (“h”) or days (“d”). For example, S85-3d resulted by soaking the S85 MBG in SBF for 3 days (i.e., 72 h). Prior to the NMR experiments, all samples were stored under dry conditions in a desiccator.

2.3. Solid-State NMR. The solid-state NMR experimentation was performed at external magnetic fields (B0) of 9.4 or 14.1 T, using Bruker Avance-III spectrometers operating at the respective 1H Larmor frequencies of ~400.1 and ~600.1 MHz. All single-pulse (“Bloch decay”) 1H NMR spectra were obtained at B0 = 9.4 T with 90° radio frequency (rf) excitation pulses at a nutation frequency of νt = 70 kHz and MBG/MBG-tSBF powders packed in either 4 mm or 7 mm ZrO2 rotors undergoing MAS at the rate νr = 7.00 kHz. The NMR acquisitions employed relaxation delays of 4.0 s and between 256 and 1024 accumulated signal transients. The NMR data of two polycrystalline HA powders (Aldrich) were recorded under similar conditions, except for using 4 mm rotors spinning at 25.1 kHz and 9.0 kHz (1H) or at 14.0 kHz for the direct 31P and indirect 1H correlation NMR spectra were recorded with spectral windows of 600.1 and 360.1 MHz.

The 2D NMR protocol shown in Figure 1c of the crystalline and amorphous phosphate phases grown in SBF, has been demonstrated that carbonate ions are present in both SBF specimens, we review our main conclusions from two characterization techniques agreed well: no significant HCA formation was detected by either method for immersion periods tiSBF < 24 h, except for S90, where 31P NMR revealed minor amounts of HCA already after 8 h. After 3 days of SBF soaking, roughly equal relative ACP:HCA fractions were observed for specimens of each S90, S85, and S58 series. Notwithstanding a net growth of ACP and HCA between 3 and 30 days, their relative fractions remained almost constant throughout, with the NMR and PXRD analyses yielding estimates of ~50% and ~60–80% of HCA, respectively, out of the total phosphate layer formed at any S90/S85/S58 MBG. It has been demonstrated that carbonate ions are present in both the crystalline and amorphous phosphate phases grown in SBF, implying that HCA forms rather than HA.

Noteworthy, the HCA formation rate is essentially independent of either the precise {Ca, Si, P} MBG composition or the textural properties—such as the surface area and pore arrangement, which differ among the S90, S85, and S58 specimens (see Table 1). This feature is likely to hold for any MBG provided that its surface area is sufficiently large (>150 m2/g); it was rationalized from the very similar silicate-reaction pathways observed for all three MBG structures, as inferred.

Table 1. MBG Compositions and Textural Properties

| Sample | CaO (mol %) | SiO2 (mol %) | P2O5 (mol %) | Stoichiometric formula | S2SBF (m2/g) | Vp (cm3 g−1) | dp (nm) | Mesoporous Structure |
|--------|-------------|--------------|---------------|------------------------|---------------|--------------|---------|-------------------|
| S90    | 10.0(9.6)   | 90.0(90.4)   | 0.0(0.0)      | Ca9.6Si90.4P0.4O190.4  | 468           | 0.63         | 5.37    | p6mm              |
| S85    | 10.0(10.6)  | 85.0(86.5)   | 5.0(2.9)      | Ca10.6Si85.0P2.9O192.5 | 480           | 0.64         | 5.38    | 1a7d              |
| S58    | 37.0(36.6)  | 58.0(59.0)   | 5.0(4.4)      | Ca37.0Si58.0P4.4O182.2 | 195           | 0.46         | 9.45    | p6mm              |

“Each MBG sample is denoted Sb, where b is the nominal oxide equivalent of SiO2 in mol % of the composition aCaO−bSiO2−cP2O5, where a + b + c = 100 mol %. Values in parentheses represent the experimentally analyzed oxide equivalents (see ref 42). Charge-balanced analyzed stoichiometric composition, with cation coefficients summing to 100.0 (mol %). Specific surface area determined by the Brunauer—Emmett—Teller method. Total pore volume calculated from the amount of N2 adsorbed at a relative pressure of P/P0 = 0.98 according to Gregg et al. Average pore diameter obtained by the Barrett—Joyner—Halenda procedure. All data are reproduced from Turdean-Ionescu et al.”

DOI: 10.1021/acs.jpcc.7b03469
J. Phys. Chem. C 2017, 121, 13223–13238
from $^{29}\text{Si}$ MAS NMR involving either direct excitation with single pulses or $^1\text{H} \rightarrow ^{29}\text{Si}$ CP $^{43}$ after a rapid leaching of Ca$^{2+}$, which is completed within the first 15–60 min of SBF soaking, essentially neat mesoporous silica remains that consequently degrades independently on the precise initial MBG surface area or pore arrangement.

As discussed in ref $43$, the main distinction among the P-bearing S85 and S58 MBGs concerns how rapidly the structure is depleted of Ca and phosphate species, which may be rationalized from subtle distinctions in their structural roles: P is depleted of Ca and phosphate species, which may be clusters $^{75}$ that readily dissolve on their contact with aqueous solutions. $^{42,43,75}$ In contrast, the CaP clusters of the S58 MBG network, whose Ca-associated silicate groups tend to accumulate near the pore-wall surface. $^{35,44}$

The latter portion only exists in Ca-rich (e.g., S90) or P-free (e.g., S90) MBGs, whereas essentially all Ca species of the S85 structure are only exists in Ca-rich (e.g., S58) or P-free (e.g., S90) MBGs, which is completed within the $r_{\text{SBF}}$. Hence, for a constant MBG mass, the resulting amount of HCA increases according to S90 < S85 $\rightarrow$ SSBF specimen, which may comprise the pristine MBG (see Table 1). Hence, for a constant MBG mass (e.g., see Section 4.2) but only to its P-free S90 counterpart. However, the property of essentially identical in vitro response mechanisms from the three distinct MBGs only applies for SBF testing under dilute conditions ($m_{\text{SBF}}$/$V$ $\leq$ 1 g/L). $^{42,43}$ Our early work employed 3–20 g/L $^{29}\text{Si}$-containing MBG samples, with $r_{\text{SBF}}$ increasing from top to bottom. All spectra reveal silicate surface-deriving resonances ($\geq$ 2 ppm), as well some narrow signals (marked by asterisks), the most prominent ones appearing at 1.1 and 3.6 ppm and stemming from CH$_3$ and OCH$_3$ groups. They originate mainly from remnant P123 block copolymers anchored at the pore-wall surface. $^{43}$ Despite that they are minor, these resonances are emphasized by their narrow peak widths ($<$ 30 Hz). These undesirable proton species are commented on further in Section 5.1, and onward we focus on the targeted $^1\text{H}$ responses from the inorganic phases.

The precise $^1\text{H}$ chemical shift ($\delta_{\text{H}}$) of each silicate/phosphate-associated OH/H$_2$O moiety is mainly dictated by its degree of hydrogen bonding to neighboring O atoms: the shift of an OH···O fragment increases when the H···O distance is decreased. $^{77,78}$ Hence, the globally lowest chemical shift ("most shielded $^1\text{H}$") is manifested by the OH resonance from HCA, $\delta_{\text{H}} \approx$ 0.05 ppm, where H-bonding is absent. $^{15}$ Naturally, this weak and broad peak is most pronounced in the NMR spectra from the MBG-30d specimens (Figure 1). Disregarding the HCA-associated $^1\text{H}$ shift and only focusing on the MBG-associated SiOH/H$_2$O ensemble, the lowest shift, $\delta_{\text{H}} \approx$ 1.85 ppm, is observed from "isolated" SiOH groups devoid of H-bonding. $^{79,80}$ they are henceforth denoted SiOH$_{\text{isol}}$. They are present at any MBG surface (e.g., see Section 4.2) but only produce clearly discernible $^1\text{H}$ resonances when the physiosorbed water content is low. Consequently, the narrow resonance at $\delta_{\text{H}} \approx$ 1.85 ppm is readily detected from each pristine MBG but is not apparent after its exposure to the aqueous solution, owing to the substantial rehydration and thereby emphasized H-bonding network among the various SiOH and H$_2$O moieties. All other $^1\text{H}$ NMR signals in the spectral region $>$ 2 ppm are relatively ill-defined for (moderately) high surface-hydration levels, due to a continuum of H-bonding scenarios and an accompanying NMR peak broadening. Yet, three main groups of $^1\text{H}$ resonances may be identified in Figure 1, and their relative contributions depend on the amount of surface-bound water: $^{44}$

(i) $^1\text{H}$ resonances in the range of 2–4 ppm, which are signatures of a "dry" silicate surface, implying a minor water adsorption and thereby weakly H-bonded OH groups. $^{79–82}$

(ii) A peak around 4.5–5 ppm that reflects physiosorbed water and more extensively H-bonded SiOH groups. $^{44,80–82}$

This ensemble comprises two distinct portions, whose NMR signals are unresolved in the $^1\text{H}$ MAS NMR of Figure 1 but are distinguishable by other NMR features: the majority of the water pool is highly mobile, while the remaining (minor) portion is bound more tightly and may constitute the inner shell of the physiosorbed species and/or water present as inclusions within the MBG pore walls. The sizable $^1\text{H} \rightarrow ^1\text{H}$ through-space dipolar interactions (see Section 4.2) of the immobile species give spinning sidebands (not shown) at the modest MAS rate $\nu_r$ = 7.00 kHz employed. $^{55,83,84}$ Yet, from the low sideband intensities relative to the centerband, we conclude that comparatively few H$_2$O molecules are immobile, whereas the dominant population does not produce sidebands but...
Figure 1. $^1$H MAS NMR spectra recorded by single pulses ($B_0 = 9.4$ T; $\nu = 7.00$ kHz) from the series of S90-$t_{\text{SBF}}$ (left panel), S85-$t_{\text{SBF}}$ (mid panel), and S58-$t_{\text{SBF}}$ (right panel) MBG specimens, with the SBF-exposure period ($t_{\text{SBF}}$) increasing from top to bottom. The results for MBG-1d are representative also for those of MBG-4h and MBG-8h (not shown). The spectra are zoomed around the main centerband signals and are normalized to equal areas across this spectral region. Asterisks mark narrow resonances around {3.6, 1.3, 1.1, 0.8} ppm that stem from OC moieties of remnant templating molecules, whereas # identifies the NMR peak associated with SiOH$_{\text{aq}}$ groups (only visible from the pristine MBGs). The gray boxes convey the approximate shift ranges representative for SiOH moieties experiencing weak, moderate, and strong H-bonding (in practice, their shift-spans overlap and are not accurately known).

Contribute to most of the centerband intensity in the spectra of Figure 1. The relative proportions of stationary and mobile water molecules depend on the precise surface hydration, but the latter portion dominates throughout (presumably ≥80%).

(iii) Weak $^1$H resonances in the high-ppm region (≥8 ppm) stem from strongly H-bonded species, encompassing minute contributions from acidic protons of HPO$_4^{2-}$ anions of ACIP$_{7,8,15}$ (see Section 4.3) but predominantly involving H-bonded SiOH ···OSi motifs, as observed from fragmented networks of hydrous silicate glasses comprising alkali/alkaline-earth metal ions.$^{35,43}$ Indeed, these NMR signals are only clearly discernible from the Ca-rich S58 MBG$^{35,43}$ and its S58-$t_{\text{SBF}}$ derivatives with short SBF-immersion periods up to ≈24 h; see Figure 1.

Noteworthy, the silicate-surface hydration and dehydration processes are reversible, and the precise $^1$H NMR peak position observed in the 3–5 ppm range reflects the given surface water content when the NMR experiment was performed.$^{44,80–82}$ Despite that the $^1$H NMR spectra presented herein were recorded on as-prepared samples stored in a desiccator prior to the NMR experiments, the quantitative proton speciations at the MBG surface are not an intrinsic sample property. Moreover, it is not possible to accurately control the amount of surface-associated water because each S90/S85/S58 specimen (and its MBG-$t_{\text{SBF}}$ counterparts) exhibits inherently distinct water affinities: the degree of water adsorption enhances concomitantly with the Ca$^{2+}$ population at the MBG surface,$^{44}$ which increases according to S85 < S90 < S58 (where the surface-associated Ca$^{2+}$ population is slightly higher for the P-free S90 structure relative to that of S85, whose entire Ca reservoir is present in the CaP clusters).$^{35,43}$ With these caveats in mind, we only discuss the gross trends of Figure 1 concerning the evolution of the proton speciations of the various samples over the 30 days of SBF immersion, where we highlight the following:

(i) All pristine S90, S85, and S58 MBGs were heated at 700 °C (see Materials and Methods) and manifest relatively “dry” surfaces revealing $^1$H NMR peak maxima at ≈4 ppm. On their SBF exposure, an enhanced surface hydration is evident for all MBG-0.25h specimens, as evidenced by the vanishing peak intensity ≈1.85 ppm from isolated silanols, accompanied by an overall resonance broadening. The elevated population of surface-adsorbed water molecules is most evident for the Ca-richest S58–0.25h specimen, which additionally manifests an NMR peak-maximum displacement from ≈3.7 ppm (pristine S58) to 5.0 ppm, the latter typical for “liquid” H$_2$O resonances.

(ii) For prolonged SBF exposure of S90 and S85 ($t_{\text{SBF}} > 24$ h), the broad NMR peak gradually splits into two primary resonances that appear around 3.4–3.7 ppm and 5.0–5.4 ppm, suggesting two main “pools” of distinct proton environments that experience weak and moderately strong H-bonding, respectively (Figure 1). Overall, for a given $t_{\text{SBF}}$ value, the S90-$t_{\text{SBF}}$ and S85-$t_{\text{SBF}}$ samples exhibit very similar NMR signatures, whereas the resonance from physisorbed water (≈5 ppm) is emphasized for the S58-$t_{\text{SBF}}$ surfaces, consistent with their higher water affinity.

(iii) In good qualitative agreement with the estimated HCA contents of Turdean-Ionescu et al.$^{42}$ (Section 3), the HCA-
associated OH resonance ($\approx 0$ ppm) is clearly visible after SBF-soaking periods $t_{\text{SBF}} > 24$ h. Its intensity grows concurrently with both the SBF-immersion period and the P content of the MBG (Table 1), i.e., along the series $S90 < S85 < S58$, which becomes particularly evident when comparing the peak intensity $\approx 0$ ppm in the NMR spectra from S90-30d and S58-30d of Figure 1.

4.2. $^1H$–$^1H$ Proximities from Double-Quantum NMR.
To qualitatively assess the spatial proximities among the various surface-associated proton species, we performed dipolar recoupling experimentation with the $[SR2]^\dagger$ pulse sequence $^{1,2,3}$ at $\nu_x = 66.0$ kHz. These experiments utilize homonuclear through-space $^1H$–$^1H$ dipolar interactions to excite 2QCs, whose buildup rate reflects the dipolar-coupling magnitude in a pair of protons. This interaction depends on the inverse cube of the $^1H$–$^1H$ internuclear distance but is also sensitive to molecular motions and averages to zero in the presence of rapid molecular reorientations. For instance, the $^1H$–$^1H$ dipolar interaction of the $CH_3$ moiety, respectively, the primary signal represents a broad autocorrelation ridge associated with hydrogen-bonded SiOH groups. Yet, a correlation is also established among two SiOH$_{\text{sol}}$ sites at the surface, as is most evident from the slice along the 1Q dimension at $\delta_1 = 3.8$ ppm, shown to the right of the 2D NMR spectrum. The S90 surface hydration level was lower when the 2Q–1Q spectrum marked by the dotted line in Figure 2. Incidentally, such “autocorrelation” signals dominate the 2D NMR spectrum from S90.

The 2Q–1Q NMR spectrum from the S90 MBG (Figure 2) is similar to our previous result from a pristine S85 specimen,$^{14}$ besides the narrow resonances at the 2QC shifts of 7.2 ppm and across 2–4 ppm that originate from 2QCs among protons within each OCH$_3$ and CH$_3$ moiety, respectively, the primary signal represents a broad autocorrelation ridge associated with hydrogen-bonded SiOH groups. Yet, a correlation is also established among two SiOH$_{\text{sol}}$ sites at the surface, as is most evident from the slice along the 1Q dimension at $\delta_1 = 3.8$ ppm, shown to the right of the 2D NMR spectrum. The S90 surface hydration level was lower when the 2Q–1Q experimentation was performed relative to that giving the NMR spectrum of Figure 1 (compare the directly excited spectra of Figures 1 and 2). Yet, considering that most silanols are weakly H-bonded (responsible for the overall largest NMR signal intensity $\approx 3.7$ ppm), the shortest $^1H$–$^1H$ distance between two SiOH$_{\text{sol}}$ moieties is expected to be longer than that involving an isolated silanol ($\delta_1 = 1.8$ ppm) and a H-bonded ($\delta_1 \approx 3.5$ ppm) counterpart. Hence, the latter correlations should also be present: while they are obscured in the 2Q–1Q spectrum by the dominating autocorrelation ridge from pairs of H-bonded SiOH groups, those NMR signals appear in the slice at $\delta_1 = 5.7$ ppm as a broad resonance extending between 2 and
4 ppm and peaking at the shift of the SiOH$_{sol}$ group (Figure 2). There is also a 2QC autocorrelation involving the resonance at $\delta_{1H} = 4.8$ ppm, whose origin is unknown; notwithstanding that both its chemical shift and narrow peak-width suggest physisorbed water, those mobile moieties should not permit 2QC excitation, as commented below.

The 2Q$^{-}$1Q NMR spectrum obtained from S58-15d is displayed in Figure 3. It overall features the same correlations as those observed from S90 but differs primarily in two aspects. First, the SBF-exposed S58-15d specimen reveals an autocorrelation peak at 0 ppm, originating from the O$	ext{H}$ groups of HCA. Second, the S58-15d powder was rehydrated prior to the NMR experiments, as manifested by the absence of a significant spectral intensity from isolated silanols in the $^1$H MAS NMR spectrum displayed in Figure 3 and by the prominent NMR peak at 5.1 ppm from mobile physisorbed water molecules. However, their motionally averaged $^1$H$^{-}$$^1$H interactions do not support 2QC excitation (see Section 4.1), as witnessed by the strong signal-intensity depletion $\gtrsim 4.5$ ppm in the projection of the 2Q$^{-}$1Q NMR spectrum of Figure 3 relative to the Bloch-decay MAS counterpart. Noteworthy, fast-MAS experimentation at $\nu_r = 66$ kHz (Figures 2 and 3) does not improve the NMR spectral resolution significantly relative to that at $\approx 10$ kHz (Figure 1) because the peak widths are mainly dictated by chemical-shift dispersion rather than by homonuclear $^1$H$^{-}$$^1$H interactions.

Despite the higher surface hydration of S58-15d compared with S90, the 2Q$^{-}$1Q NMR spectrum reveals that SiOH$_{sol}$ surface groups are present (Figure 3). Yet, as expected, their associated $^1$H resonances are weaker and appear to mainly involve correlations with H-bonded silanols; see the slices extracted at $\delta_{2Q} = 4.2$ ppm and $\delta_{2Q} = 5.7$ ppm. Moreover, the larger population of H-bonded silanols is manifested in the 2Q$^{-}$1Q spectrum by the autocorrelation ridge extending over a larger shift range in both spectral dimensions (which is responsible for the “tail” toward higher shifts observed in the projection).

### 4.3. Phosphate-Associated Proton Environments

As follows from the hitherto discussed $^1$H NMR results, only a minority of the $^1$H resonances derive from the biomimetic ACP/HCA layer, most merely being associated with silanols and physisorbed water molecules at the MBG surface. Consequently, we performed $^1$H$\rightarrow$$^{31}$P CPMAS NMR experimentation for selectively probing the protons in close spatial proximity to phosphate groups of the heterogeneous calcium phosphate layer. Figure 4 displays a $^1$H$^{-}$$^{31}$P 2D HETCOR NMR spectrum recorded from the S85-1d sample. It informs which proton and phosphate species are close in space by a pairwise correlation appearing at the 2D coordinate {δ$_{1H}$, δ$_{31P}$}, with the chemical shifts of $^1$H (δ$_{1H}$) and $^{31}$P (δ$_{31P}$) being encoded along the vertical and horizontal dimensions of the 2D NMR spectrum, respectively. Each corresponding 2D spectral projection reveals a $^1$H (vertical) and $^{31}$P (horizontal) spectrum solely manifesting resonances that convey sufficiently strong $^1$H$^{-}$$^{31}$P contacts, with a “strong” (“weak”) $^1$H$^{-}$$^{31}$P contact implying a short (long) $^1$H$^{-}$$^{31}$P internuclear distance and/or a higher (lower) number of protons in the vicinity of $^{31}$P.

The $^{31}$P projection in Figure 4 exhibits a broad and nearly Gaussian peak at δ$_{p} = 3.05$ ppm. It comprises three main components, all centered around nearly identical $^{31}$P chemical shifts, as illustrated by the slices extracted at δ$_{1H} = \{0.0, 5.8, 12.0\}$ ppm from the 2D HETCOR NMR spectrum. One narrow $^{31}$P NMR peak of full width at half-maximum (fwhm) height of 1.94 ppm (315 Hz at $B_0 = 9.4$ T) is correlated with the narrow OH signal appearing at δ$_{1H} = 0.0$ ppm; it stems from the orthophosphate groups of HCA$^{27,28}$ that represents the well-crystalline “core” in the core−shell structural picture (see Section 5.2). All other $^{31}$P NMR responses are significantly
broader (fwhm 5.1−5.4 ppm) and correlate with 1H shifts δH > 4 ppm, all of which originate from ACP,16,28−59 i.e., the “hydrated HCA surface” in the core−shell view;16,17,65,66 the most intense NMR peak at the shift pair (δH, δP) = (5.8, 3.0) ppm is assigned to “nonapatitic” PO4−3 groups nearby water molecules, whereas the remaining correlations with δH ≥ 9 ppm stem mainly from P−OH contacts in HPO42− anions. These NMR responses account for the broad “tail” toward higher δH values of the 2D HETCOR spectrum. Notably, they are not visible in the directly excited NMR spectra of Figure 1, underscoring that the ACP-associated 31P HETCOR 2D NMR spectrum (left panel) recorded from S85-1d at T1 = 0.9 s, 0.3 nm 31P distances, respectively. As discussed previously,28 the overall stronger 1H−31PO4− contacts of ACP relative to HCA are mirrored in its NMR signal being maximized already at τCP ≈ 1.5 ms, whereas the HCA counterpart exhibits a slower but continuous growth for increasing contact periods, reaching a plateau around 6 ms. Overall, the most rapid magnetization transfers occur across the P−OH fragments of HPO42− groups:16 this accounts for the generally emphasized high-ppm intensities in the diffCP-derived data of Figure 5 that utilized τCP = 0.2 ms, as compared with the 1H−31P HETCOR counterpart of Figure 4 that employed a longer contact period of 1.0 ms and thereby favoring the H2O/PO42− pair.

Consequently, utilizing a short contact interval of 0.2 ms emphasizes the 1H NMR responses from the amorphous phase(s) and enables their (near) selective probing. This is evident from Figure 5: regardless of the τCP value, all NMR results for the MBG-4h samples stemming from a short SBF-exposure interval reveal predominantly resonances from ACP. Yet, the very weak OH peak intensity ≈0 ppm observed for τCP = 6.0 ms evidences a minute HCA formation already at this short incubation period (see Section 5.4). Moreover, the nearly complete selective excitation of the 1H NMR signals from ACP is manifested by all NMR spectra acquired with τCP = 0.2 ms from the MBG-30d specimens, which comprise both ACP and HCA (Figure 5). For the MBG-30d samples, all NMR data recorded by employing the longer contact period (τCP = 6.0 ms) reveal mainly the narrow OH signal from HCA because relaxation processes during CP damp the ACP-stemming resonances even over short contact intervals of a few milliseconds.16,17,28

Figure 4. 1H−31P HETCOR 2D NMR spectrum (left panel) recorded from S85-1d at B0 = 9.4 T and ν1 = 10.00 kHz (τCP = 1.0 ms) and shown together with its projections along the 31P (horizontal; top) and 1H (vertical; right) spectral dimensions. The right panel displays slices along the 31P dimension, corresponding to PO42−/OH correlations of HCA (δH = 0.0 ppm), as well as nonapatitic PO42−/H2O (δH = 5.8 ppm) and HPO42− (δH = 12.1 ppm) contacts of ACP. The 31P fwhm values are given at the right spectral portions of the slices. The lowest 2D contour level is at 6% of the maximum amplitude.
Besides the targeted $^1\text{H} \rightarrow ^{31}\text{P}$ magnetization transfers from the protons of the ACP/HCA components, weak transfers also occur from those of the organic templating molecules; they are most pronounced for the MBG-4h specimens for $r_{CP} = 6.0$ ms. We consider these signals as artifacts that might have leaked through the phase cycle, although their emphasized intensities consistently observed for the longer contact interval of 6.0 ms may indeed reflect transfers from the aliphatic surface-anchored organic moieties to the surrounding ACP phase growing at the pore surface.

5. DISCUSSION

5.1. Origin of the $^1\text{H}$ Signals around 1 ppm? The narrow resonances at 0.8, 1.1, and 1.3 ppm observed in $^1\text{H}$ NMR spectra of the MBG and MBG-$r_{SBF}$ samples (Figures 1–3 and 5) are doubtlessly of organic origin. Besides the results herein, we refer to Leonova et al.44 for detailed assignments and further discussions. Concerning $^1\text{H}$ NMR characterizations of the already complex systems of mesoporous silica coexisting with its heterogeneous surface layer of calcium phosphates, these NMR peaks are a nuisance and may lead to assignment ambiguities—even as to the precise organic source molecules, as shown below. However, these NMR signals are worth discussing in more depth because similar (but distinct) NMR responses are frequently observed across the shift range 0.8–1.4 ppm from apatites, encompassing HA,8,16,22 HCA,6,13,14,19–21 fluoro-hydroxyapatite,15,87 as well as bone mineral.6,13,14 These resonances are usually very weak from well-crystalline HA, as may be verified from the $^1\text{H}$ NMR spectra of Figure 6(a, b).

First focusing on the minor $^1\text{H}$ resonances from synthetic H(C)A, their precise shifts and intensities vary slightly among samples and studies, but (at least) two peaks are reported around 0.8–0.9 ppm and 1.2–1.4 ppm, respectively, occasionally accompanied by another resonance around 2.0 ppm.16,21 Their narrow peak widths ($\lesssim 30$ Hz) and absence of magnetization transfers in $^1\text{H} \rightarrow ^{31}\text{P}$ CPMAS-based experimentation suggest species that are either highly mobile and/or remote from phosphate groups.14,16 Furthermore, the peak intensities grow concurrently with the surface area of the H(C)A crystallites and are most pronounced for nanocrystalline HA powders.16 Altogether, these characteristics point toward surface-associated species whose populations depend on the surface hydration level, but unambiguous assignments have until recently remained elusive. Similar narrow peaks are also observed from HCA with significant carbonate-for-phosphate substitutions ("B-type" HCA),13,18–21 where they have been attributed to OH groups close to CO$_3^{2-}$ ions.13,16,19

The hitherto most convincing solution to the enigma of the origin of the narrow NMR responses in the 0.8–1.4 ppm range—at least for well-ordered HA—was recently provided by Ben Osman et al.,22 who applied a suite of characterization protocols to HA samples with controlled (but relatively low) surface hydration levels. All $^1\text{H}$ resonances appearing at {0.8, 1.1, 1.3} ppm were attributed to H$_2$O molecules that terminate the OH channels (which are aligned with the $c$ axis of the crystal frame). The 1.1 ppm signal dominates for higher surface hydration levels. All $^1\text{H}$ resonances appearing at {0.8, 1.1, 1.3} ppm were attributed to H$_2$O molecules that terminate the OH channels (which are aligned with the $c$ axis of the crystal frame). The 1.1 ppm signal dominates for higher surface hydration levels. All $^1\text{H}$ resonances appearing at {0.8, 1.1, 1.3} ppm were attributed to H$_2$O molecules that terminate the OH channels (which are aligned with the $c$ axis of the crystal frame). The 1.1 ppm signal dominates for higher surface hydration levels.

Figure 5. $^1\text{H}$ NMR spectra of the indicated MBG-4h (top panel) or MBG-30d specimens (bottom panel); they were recorded either directly by single pulses ("1 pls") or by the $^1\text{H} \rightarrow ^{31}\text{P}$ "diffCP" protocol,17 the latter labeled by the respective contact interval ($r_{CP}$) of 0.2 ms or 6.0 ms used for $^1\text{H} \rightarrow ^{31}\text{P}$ CPMAS. The NMR spectra are normalized to equal areas. The broad resonances, emphasized in the NMR spectra obtained with $r_{CP} = 0.2$ ms and peaking at $\approx 5$ ppm and $\approx 12$ ppm stem from H$_2$O and HPO$_4^{2-}$ moieties, respectively. The weak but sharp peak at 10 ppm (marked by an asterisk) is attributed to the HPO$_4^{2-}$ group of brushite.11,13,15

Perspective 1

Perspective 2

Perspective 3
5.2. Disordered Phosphate Component: ACP or Apatite Surface Layer? A "core−shell" structural model, involving a crystalline HCA core and a disordered surface layer rich in "nonapatitic" HPO$_4^{3−}$ moieties, has been proposed and discussed in the literature.$^{10,50,51,65−66}$ Recently, this structural picture of H(C)A appears to be well accepted, partially thanks to the recent and detailed NMR-derived constitution of both synthetic H(C)A and biological apatite.$^{8,16,23,24}$ Besides the Ca$^{2+}$ constituent of both core−shell components (and CO$_3^{2−}$ in the context of HCA), the model involves an ordered H(C)A core of "apatitic" PO$_4^{3−}$ and OH$^{−}$ ions and a disordered surface layer comprising water and "nonapatitic" HPO$_4^{2−}$ and PO$_4^{3−}$ moieties, as illustrated in Figure 7(a).

Unfortunately, different nomenclatures encountered in the literature concerning the "hydrated surface layer" and "ACP" obscure their strong similarities, thereby implicitly highlighting their distinctions. Nevertheless, common to all recently reported (approximate) HCA-surface compositions$^{8,16,23,24}$ is the presence of HPO$_4^{2−}$ and H$_2$O species, a feature also shared with both OCP$^{39,15}$ and recently deduced compositions of ACP.$^{36,56}$ Moreover, the demonstrated variable H$_2$O contents in both ACP and the surface layer of synthetic/biological HCA$^{36,56}$ imply an inherently nonunique chemical speciation that depends on the precise surface hydration level. The $^{31}$P HETCOR data of ref 8 obtained from both synthetic HCA and sheep bone are consistent with a "dry" and "wet" HCA surface reflecting a comparatively enhanced contribution from HPO$_4^{3−}$ and PO$_4^{3−}$/H$_2$O species, respectively. (Analogously with the direct dependence of the $^1$H speciation at the MBG silicate core/surface on its hydration level; see Section 4.1.) Likewise, the $^{31}$P HETCOR NMR spectra monitoring the transformation between disordered/HA phases in liposomes$^{38}$ are readily rationalized by ACP incarnations with variable H$_2$O/HPO$_4^{2−}$ contents as the crystallization progressed (compare with the results by Wang et al.$^8$). On the basis of different $^{31}$P CPMAS parameters, Chen et al.$^{59}$ deliberately chose the nomenclature "disordered phosphate" to highlight its distinction to "ACP" obtained directly by precipitation. Nonetheless, it may be too early to dismiss its identification with ACP, where we also note that distinct and sequential "ACP-1" and "ACP-2" precursors of HA are discussed in the literature (e.g., see refs 54 and 57).

Furthermore, despite careful and sensible analyses,$^{7,8,16,23,24}$ the nonquantitiveness of the NMR experimentation invoked for discriminating and estimating the various proton/phosphate populations in the "core" and "surface" apatite domains implies relatively large uncertainties that merit caution in claiming precise compositions. The hurdles of precisely quantifying the relative PO$_4^{3−}$, HPO$_4^{2−}$, and H$_2$O contents of "ACP" and "OCP-like" phases, coupled with the nonetheless similar compositions reported for the H(C)A surface portion,$^{8,16,23,24,67}$ suggest that "ACP" is a good approximant. Hence, it may be premature to make too categorical statements when describing the nature of the H(C)A surface layer, as well as the "disordered calcium phosphate" phase observed in liposomes.$^{59}$ Yet, with one notable exception,$^8$ the H(C)A surface layer is in general not identified explicitly as "ACP".$^{4,10,16,50,65}$ Moreover, adopting a looser terminology of "disordered OCP" would better bring out its structural similarity with both ACP and the H(C)A surface layer.

Concerning the issue of "separate HCA and ACP components" or an "HCA core and a disordered surface layer", one must distinguish the aspects of (i) the core/surface
parts of each individual HCA particle [Figure 7(a)] from (ii) a
distribution of such particles with variable surface and core
volume fractions [i.e., an ensemble of particles with varying
degrees of crystallinity; see Figure 7(b)], yet noting that they
are in general correlated. For instance, since the crystallization of
in vitro formed HCA starts within the ACP phase, its
interior will have a comparatively higher fraction of crystallites
where the “HCA core” dominates over its surface (ACP) portion, while the latter prevails in the outer parts of the bulk;
see Figure 7(c). This relates to the inferences of Beniash et al.,
who attributed the “outer” and “inner” parts of mouse
enamel to ACP and HCA, respectively. However, their
interpretation did not refer specifically to the core/surface of
individual HCA crystallites but merely to large collections
thereof probed over a 102–103 nm scale. Likewise, the rat
dentin model proposed by Chan and co-workers was
discussed in terms of inner (HCA) and outer (ACP) portions.

Which most naturally adopted viewpoint of an “ACP phase”
or an “apatite surface layer” depends on the relative amounts of
crystalline and amorphous material, where we here also comment on our interpretations in the context of the biomimetic phosphate layer of the MBG-τSBF specimens: In
the limit of a negligible apatite core, i.e., when the volume of
the HCA surface layer vastly dominates, it seems most reasonable to view the particle ensemble as an ACP phase coexisting with a low number of small apatite crystallites
[Figure 7(c)]; this applies for MBGs immersed in SBF for
periods τSBF < 24 h, where the specimen remains XRD
amorphous and the HCA (“core portion”) is very minor. In
contrast, for more mature nanocrystalline H(C)A/bone mineral
with the surface layer accounting for ≤50% in volume —
relevant for the present MBG-τSBF specimens with τSBF ≥3
days —the core–shell picture may be most suitable [Figure
7(d)], yet with the identification of the “surface layer” as
“ACP,” the latter taken to encompass a range of chemical compositions. Furthermore, the “surface layer” observed for
nanocrystalline HCA particles is absent for well-ordered
and micrometer-sized HA crystals prepared in vitro by precipitation followed by heat treatment; see Figure 6(a) for the 1H
NMR signatures of such a specimen. This feature may be viewed as a complete ACP→HA conversion.

Worth highlighting is that once an equivalence between the
HCA surface layer and ACP is accepted, the controversy of
ACP being a component of (mature) bone mineral is settled,
while it also becomes (even) more plausible that ACP would
indeed be a precursor of biological apatite. Notably, this
reconciles the viewpoints that “ACP precedes HCA formation”
or “small and poorly ordered HCA particles form directly”,
which are simply two sides of the same coin, with their seeming
differences becoming semantic.

5.3. Proton Environments of in Vitro Grown ACP and
HCA. The 1H NMR data of Figure 5 corroborate our earlier
inferences from 31P NMR about a dominance of ACP after 4 h
of SBF exposure, whereas both ACP and HCA coexist after 30
days of MBG exposure to SBF.22 The 1H–31P results of Figures
4 and 5 accord overall with our previous 1H–31P HETCOR 2D
NMR spectra27,28 from SBF-soaked S85 specimens that featured very similar analyzed cation compositions as the present one, as well as those reported for synthetic nanocrystalline H(C)A specimens and bone/dentin.6,8,13,16,23,24

However, there is one notable distinction to our earlier results: 1H resonances from acidic protons were not observed,
and structural water represented the sole proton reservoir of
ACP.27,28 The signal-to-noise ratios (S/N) of the present NMR
data (notably so the 1H–31P HETCOR NMR spectra) are
significantly higher than those of our previous 1H–31P HETCOR results, suggesting that the broad resonances in
the high-ppm spectral region might have escaped detection.
This is, however, unlikely considering that the H2O NMR peak
was readily observed,27,28 implying that the 1H resonances from
acidic sites should also have been detected, if present in
comparable H2O/HPO42− proportions as those revealed in
Figures 4 and 5. More probable is that the ACP component of
our present SBF-exposed MBG specimens comprises signifi-
cantly higher HPO42− populations (except for S90-4h, see
Figure 5).
The apparent distinct ACP compositions could potentially stem from the more dilute MBG loading employed in our current SBF testing relative to that of previous reports, which altered the reactions at the MBG surface and thereby also the HCA growth\(^1\),\(^2\),\(^3\),\(^4\),\(^5\),\(^6\) (Section 3). However, considering that the precise ACP composition depends on the net hydration level\(^7\) (Section 5.2), it is more likely that the biomimetic phosphate layers of the present MBG-\(\tau_{\text{SBF}}\) specimens were less hydrated than the previous ones (refs 27 and 28). This readily rationalizes that the amount of acidic protons is emphasized relative to that of \(\text{H}_2\text{O}\) molecules after 30 days of SBF immersion compared with the ACP phase formed after 4 h, as follows by contrasting the \(^1\text{H}→\text{P}\) diffCP NMR results (\(\tau_{\text{CP}} = 0.2\) ms) of Figure 5 for the short and long SBF-soaking periods.

At a fixed \(\tau_{\text{SBF}}\)-value of either 4 h or 30 days, the \(^1\text{H}\) speciation of each biomimetic ACP/HCA component is almost from each single-pulse \(^31\text{P}\) MAS NMR spectra. Noteworthy, the well as the more recently introduced option of deconvoluting using infrared spectroscopy, PXRD, or electron microscopy, as suggests that di... order. The main distinction observed among the diffCP-derived \(^1\text{H}\) NMR spectra in Figure 5 is the absence of signals from acidic protons in the S90-4h specimen. Overall, the significant shift dispersion for all \(\text{H}_2\text{O}/\text{HPO}_4^{2-}\) resonances from ACP reflect highly disordered \(^1\text{H}\) environments. Yet, some ordering tendencies are evident from the \(^1\text{H}\) NMR spectra of the MBG-30d specimens relative to their MBG-4h counterparts (\(\tau_{\text{CP}} = 0.2\) ms): at the longer SBF-immersion period, a well-defined NMR peak maximum \(\approx 12\) ppm is observed for the \(\text{HPO}_4^{2-}\) resonances. Incidentally, this shift agrees with that around 12–13 ppm reported for the acidic protons of OCP\(^7\),\(^8\),\(^9\),\(^10\),\(^11\) suggesting “disordered OCP” environments. The S85-30d sample additionally manifests a very weak and sharp peak at \(\delta_{\text{H}} = 10\) ppm, indicating a minute brushite (\(\text{CaHPO}_4·2\text{H}_2\text{O}\)) formation.\(^11\),\(^12\),\(^13\) Moreover, while the biomimetic HCA phase in the MBG-30d specimens is classified as “crystalline,” a remaining structural disorder—partly stemming from incorporation of \(\text{CO}_3^{2-}\) and \(\text{Na}^+\) ions\(^13\),\(^14\) is mirrored in the

**5.4. Detecting the Onset of HCA Formation: \(^1\text{H}\) versus \(^31\text{P}\) NMR.** The reduced experimental time offered by \(^1\text{H}→\text{P}\) diffCP/MAS NMR experimentation relative to that of arranging a full 2D HETCOR or \(^31\text{P}→\text{H}\) CPMAS NMR spectrum, suggests that diffCP is an attractive alternative for detecting the onset of HCA formation, besides the standard techniques of using infrared spectroscopy, PXRD, or electron microscopy, as well as the more recently introduced option of deconvoluting single-pulse \(^31\text{P}\) MAS NMR spectra.\(^27\),\(^28\) Noteworthy, the clearly discernible HCA-associated \(^1\text{H}\) resonance observed from each S90-4h, S85-4h, and S88-4h sample (Figure 5) unambiguously evidences that a minute but non-negligible HCA formation occurred already after 4 h of SBF exposure. This may be contrasted with the failure of PXRD to detect HCA from any of the present MBG specimens with \(\tau_{\text{SBF}} < 24\) h. The same conclusion was reached by \(^31\text{P}\) NMR, except that the P-free S90 MBG revealed HCA after 8 h of SBF exposure.\(^42\) The \(^1\text{H}\) resonance intensity (Figure 5; \(\tau_{\text{CP}} = 0.2\) ms) grows along the series S90 < S85 < S88, in accordance with the results of Figure 1 that corroborate our previous conclusion from \(^31\text{P}\) NMR.\(^42\)

The discrepancy with \(^31\text{P}\) NMR—which suggested a shorter HCA induction period of 8 h from the S90 MBG relative to 24 h for S85 and S88\(^42\) (in contrast with the \(^1\text{H}\) NMR data herein)—stems partially from interferences of broad \(^31\text{P}\) resonances from remnants of the amorphous CaP clusters of the pristine S88/SS8 MBG structures: while they dissolve readily into the aqueous medium, this process is likely not completed until \(\approx 8−24\) h (and is slower for S88 relative to S85\(^42\),\(^43\)). The quantification of each biomimetic ACP and HCA component by \(^31\text{P}\) NMR relies on the overall peak-narrowing accompanying the ACP → HCA transformation and a deconvolution of the \(^31\text{P}\) NMR signal into two components, one broad from ACP and one narrow from HCA, both of which overlap and are centered around the same \(^31\text{P}\) chemical shift around 3 ppm;\(^27\),\(^42\),\(^60\) see Figure 4. However, the \(^31\text{P}\) NMR signals from the disordered CaP clusters are essentially indistinguishable from the SBF-induced ACP component.\(^27\),\(^28\),\(^42\) Consequently, if they are present together, the content of the (as-assumed sole) ACP phase becomes overestimated, with the CaP-stemming \(^31\text{P}\) resonances obscuring the detection of the narrow \(^31\text{P}\) counterpart from HCA: the absence of broadening of the \(\text{net} \text{NMR peakshape from the CaP clusters most likely enabled the detection of HCA from the P-free S90 specimen already after 8 h in SBF, notwithstanding that larger amounts of HCA are formed from the P-bearing S85 and (particularly) SS8 MBGs over longer exposure periods,\(^42\) as commented above. Consequently, another factor contributing to the apparently delayed HCA generation from the S85/SS8 MBGs is the presence of higher amounts of ACP formed from the P-bearing MBGs, accomplished by a larger contribution of broad \(^31\text{P}\) resonances. Fortunately, the undesirable CaP-stemming NMR signals are absent when exploiting \(^1\text{H}\) NMR, although the inherently nonquantitative nature of CPMAS-based experimentation makes accurate quantifications cumbersome. Hence, once the HCA formation is significant, we recommend single-pulse \(^31\text{P}\) NMR for quantifying the relative ACP/HCA fractions; we guide the reader to refs 42 and 60 for discussions on the relative merits of PXRD and \(^31\text{P}\) MAS NMR for detecting and quantifying the relative and absolute ACP/HCA amounts. We stress that in the core—shell HCA picture (Section 5.2), the “HCA fraction” deduced from \(^31\text{P}\) NMR reflects the well-ordered core of the HCA particles, whereas for an essentially complete ACP → HCA conversion, the “ACP fraction” is best interpreted as the volume fraction of the disordered surface of the HCA crystallites.

**6. CONCLUSIONS.** We have examined the various H species present at the surfaces of three pristine MBGs, as well as in the silicate/phosphate portions of the heterogeneous MBG-\(\tau_{\text{SBF}}\) specimens resulting after immersing each MBG in SBF for variable periods up to 30 days. \(^1\text{H}\) NMR spectra recorded by single pulses are dominated by resonances from the MBG surface, encompassing two distinct pools of physiosorbed water molecules: a main population of mobile species and a minor ensemble that is more strongly surface bound; their precise amounts are difficult to quantify and depend on the overall surface hydration level. The MBG surface is also rich in SiOH moieties that exhibit variable degrees of H-bonding, distinguished by their \(^1\text{H}\) chemical shifts, where three coexisting proton species were identified: those of (i) “isolated silanols” devoid of H-bonding and those experiencing (ii) weak and (iii) strong H-bonding. Their relative abundances depend on the MBG-surface hydration...
level, where weakly H-bonded SiOH groups are most common at surfaces of the Ca-poor (pristine) S90/S85 MBGs and their MBG-rSBF counterparts resulting from extended SBF-soaking periods beyond 24 h. For short SBF-exposure intervals within a few hours, on the other hand, a higher surface hydration level is manifested by both the (near) absence of isolated SiOH groups and an overall resonance broadening. Protons of the strongest H-bonded motifs, such as SiOH···OSi, are only observed in the fragmented silicate networks of the Ca-rich—and thereby most hydrophilic—S58 MB surface and its SBF-soaked counterparts. 2Q–1Q correlation 1H NMR experiments revealed predominantly autocorrelations among the H-bonded SiOH/H2O moieties, as well as between pairs of isolated silanols.

In directly excited 1H NMR spectra, resonances from the minor ACP/HCA components are generally swamped by those from their MBG counterparts. Nevertheless, over days of SBF soaking, the HCA-characteristic OH signal (≈50 ppm) is clearly discernible. Its intensity grows concomitantly with the soaking period and the P content of the (pristine) MBG, i.e., along the series S90 < S85 < S58, thereby corroborating the HCA-formation trends deduced by 31P NMR from the same specimens.42 Yet, an accurate probing of the minor H speciations of the biomimetic ACP/HCA components of the MBG-rSBF specimens is best performed with heteronuclear 1H–31P CPMAS-based experimentation, which revealed distinctly different 1H reservoirs of the amorphous and crystalline phosphate portions: the latter comprises solely OH groups, whereas ACP incorporates water and acidic protons of HPO4 2− anions. Besides the strong P–OH contacts within HPO4 2− species, 2D HETCOR NMR revealed contacts between “nonapatitic” PO4 3− ions and water molecules. Hence, in accordance with mineralization studies employing direct HCA precipitation from solution,8,26–58 the ACP phase initially formed at the MBG surface in SBF comprises H2O, PO4 3−, and HPO4 2− species (besides Ca2+ and CO3 2− ions).57 A minor ordering of ACP was observed after 30 days of MBG exposure to SBF relative to that after 4 h, as witnessed by slightly better resolved 1H NMR signals from the H2O and HPO4 2− species.

Whenever the primary focus is on the 1H environments of the biomimetic phosphate layer, we stress the advantages of using the 1H-detected double-resonance 1H–31P MAS experimental protocol of ref 17 relative to either a full 2D HETCOR NMR acquisition or time-consuming 31P → 1H CPMAS.14,85,86 Surprisingly, the utility of this technique appears to be overlooked in the community. Besides that 1H–31P double-resonance NMR experimentation offers more rapid NMR acquisitions than single-pulse 31P NMR, we also demonstrated that it is a more sensitive tool for detecting the onset of HCA formation, notably so from P-bearing (M)BGs at short SBF-exposure periods, where the 31P resonances of the glass overlap with those from the biomimetic phases, thereby increasing the net peakwidth and obscuring the narrow but very weak NMR signal from HCA.

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: mattias.eden@mmk.su.se.*

**ORCID**

Baltzar Stevensson: 0000-0001-7109-5068

Mattias Edén: 0000-0001-9409-2601

**Present Address**

‡Department of Chemistry, New York University Abu Dhabi, Abu Dhabi, United Arab Emirates.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the Swedish Research Council (projects VR-N'T (projects VR-NT 2010-4943 and 2014-4667) and by the Ministerio de Economía y Competitividad, Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER) (projects MAT 2013-43299-R, 2015-64831-R, and 2016-75611-R, AEI/FEDER, UE). M.V.-R. acknowledges funding from the European Research Council (Advanced Grant VERDI; ERC-2015-AdG Proposal 694160). C.T.-I. was supported by a postdoctoral grant from the Carl Trygger Foundation.

**REFERENCES**

(1) Weiner, S.; Wagner, H. D. The Material Bone: Structural-Mechanical Function Relations. *Annu. Rev. Mater. Sci.* 1998, 28, 271–298.

(2) Olszta, M. J.; Cheng, X.-G.; Jee, S. S.; Kumar, R.; Kim, Y.-Y.; Kaufman, M. J.; Douglas, E. P.; Gower, L. B. Bone Structure and Formation: A New Perspective. *Mater. Sci. Eng., R* 2007, 58, 77–116.

(3) Traub, W.; Arad, T.; Weiner, S. Three-Dimensional Ordered Distribution of Crystals in Turkey Tendon Collagen Fibers. *Proc. Natl. Acad. Sci. U. S. A.* 1989, 86, 9822–9826.

(4) Rey, C.; Combes, C.; Drouet, C.; Glüümch, M. J. Bone Mineral. Update on Chemical Composition and Structure. *Osteoporos Int.* 2009, 20, 1013–1021.

(5) Hu, Y.-Y.; Rawal, A.; Schmidt-Rohr, K. Strongly Bound Citrate Stabilizes the Apatite Nanocrystals in Bone. *Proc. Natl. Acad. Sci. U. S. A.* 2010, 107, 22425–22429.

(6) Wilson, E. E.; Awonsusi, A.; Morris, M. D.; Kohn, D. H.; Tecklenburg, M. M. J.; Beck, L. W. Three Structural Roles of Water in Bone Observed by Solid-State NMR. *Biophys. J.* 2006, 90, 3722–3731.

(7) Hu, Y.-Y.; Liu, X. P.; Ma, X.; Rawal, A.; Prozorov, T.; Akinc, M.; Mallapragada, S. K.; Schmidt-Rohr, K. Biomimetic Self-Assembling Copolymer-Hydroxyapatite Nanocomposites with the Nanocrystal Size Controlled by Citrate. *Chem. Mater.* 2011, 23, 2481–2490.

(8) Wang, Y.; von Eeuw, S.; Fernandes, F. M.; Cassaignon, S.; Selmane, M.; Laurant, G.; Pehau-Arnaudet, G.; Coelho, C.; Bonhomme-Coury, L.; Giraud-Guille, M.-M.; et al. Water-Mediated Structuring of Bone Apatite. *Nat. Mater.* 2013, 12, 1144–1153.

(9) Davies, E.; Müller, K. H.; Wong, W. C.; Pickard, C. J.; Skepper, J. N.; Duer, M. J. Citrate Bridges between Mineral Platelets in Bone. *Proc. Natl. Acad. Sci. U. S. A.* 2014, 111, E1354–E1363.

(10) Combes, C.; Cazalbou, S.; Rey, C. Apatite Biominerals. *Minerals* 2016, 6, 1–25.

(11) Santos, R. A; Wind, R. A.; Bronnimann, C. E. 1H CRAMPS and 1H–31P Heteronuclear Experiments on Bone, Bone Mineral and Model Calcium Phosphate Phases. *J. Magn. Reson., Ser. B* 1994, 105, 183–187.

(12) Cho, G.; Wu, Y.; Ackerman, J. L. Detection of Hydroxyl Ions in Bone Mineral by Solid-State NMR Spectroscopy. *Science* 2003, 300, 1123–1127.

(13) Källak-Hachulska, A.; Samoson, A.; Kolodziejski, W. 1H MAS and 1H→31P CP/MAS NMR Study of Human Bone Mineral. *Calciif. Tissue Int.* 2003, 73, 476–486.

(14) Kolmas, J.; Kolodziejski, W. Concentration of Hydroxyl Groups in Dental Apatites: A Solid-State 1H MAS NMR Study Using Inverse 31P→1H Cross-Polarization. *Chem. Commun.* 2007, 4390–4392.

(15) Yesinowski, J. P.; Eckert, H. Hydrogen Environments in Calcium Phosphates: 1H MAS NMR at High Spinning Speeds. *J. Am. Chem. Soc.* 1987, 109, 6274–6282.
Correlations in Amorphous Solids. Phys. Chem. Chem. Phys. 2008, 10, 6635–6644.
(72) Teymoori, G.; Pahari, B.; Stevenson, B.; Edén, M. Low-Power Broadband Homonuclear Dipolar Recoupling Without Decoupling: Double-Quantum $^{13}$C NMR Correlations at Very Fast Magic-Angle Spinning. Chem. Phys. Lett. 2012, 547, 103–109.
(73) Teymoori, G.; Pahari, B.; Edén, M. Low-Power Broadband Homonuclear Dipolar Recoupling in MAS NMR by Two-Fold Symmetry Pulse Schemes for Magnetization Transfers and Double-Quantum Excitation. J. Magn. Reson. 2015, 251, 205–220.
(74) States, D. J.; Haberkorn, R. A.; Ruben, D. J. A Two-Dimensional Nuclear Overhauser Experiment with Pure Absorption Phase in Four Quadrants. J. Magn. Reson. 1982, 48, 286–292.
(75) Mathew, R.; Turdean-Ionescu, C.; Stevenson, B.; Izquierdo-Barba, I.; García, A.; Arcos, D.; Vallet-Regí, M.; Edén, M. Direct Probing of the Prophosphate-Ion Distribution in Bioactive Silicate Glasses by Solid-State NMR: Evidence for Transitions between Random/Clustered Scenarios. Chem. Mater. 2013, 25, 1877–1885.
(76) Stevenson, B.; Mathew, R.; Edén, M. Assessing the Phase Distribution in Bioactive Phosphosilicate Glasses by $^{31}$P Solid-State NMR and MD Simulations. J. Phys. Chem. B 2014, 118, 8865–8876.
(77) Berglund, B.; Vaughan, R. W. Correlations Between Proton Chemical Shift Tensors, Deuteron Quadrupole Couplings, and Bond Distances in Solids. J. Chem. Phys. 1980, 73, 2037–2043.
(78) Brunner, E.; Stemberg, U. Solid-State NMR Investigation of the Nature of Hydrogen Bonds. Prog. Nucl. Magn. Reson. Spectrosc. 1998, 32, 21–57.
(79) Bronnimann, C. E.; Ziegler, R. C.; Maciel, G. E. Proton NMR Study of Dehydration of the Silica Gel Surface. J. Am. Chem. Soc. 1988, 110, 2023–2026.
(80) Liu, C. C.; Maciel, G. E. The Fumed Silica Surface: A Study by NMR. J. Am. Chem. Soc. 1996, 118, 5103–5119.
(81) Grünberg, B.; Emmler, T.; Gedat, E.; Shenderovich, I.; Findenegg, G. H.; Limbach, H.-H.; Bunkowsky, G. Hydrogen Bonding of Water Confined in Mesoporous Silica MCM-41 and SBA-15 Studied by $^1$H Solid-State NMR. Chem. – Eur. J. 2004, 10, 5689–5696.
(82) Trébosc, J.; Wiensch, J. W.; Huh, S.; Lin, V. S.-Y.; Pruski, M. Solid-State NMR Study of MCM-41-Type Mesoporous Silica Nanoparticles. J. Am. Chem. Soc. 2005, 127, 3057–3068.
(83) Kohn, S. C.; Dupree, R.; Smith, M. E. Proton Environments and Hydrogen-Bonding in Hydrous Silicate Glasses from Proton NMR. Nature 1989, 337, 539–541.
(84) Robert, E.; Whittington, A.; Fayon, F.; Pichavant, M.; Massiot, D. Structural Characterization of Water-Bearing Silicate and Aluminosilicate Glasses by High-Resolution Solid-State NMR. Chem. Geol. 2001, 174, 291–305.
(85) Crosby, R. C.; Reese, R. L.; Haw, J. F. Cross Polarization Magic Angle Spinning Proton NMR Spectroscopy of Solids. J. Am. Chem. Soc. 1988, 110, 8550–8551.
(86) Itohe, T.; Nakamura, S.; Nemoto, R.; Senna, M. Solid-State Double Nuclear Magnetic Resonance Study of the Local Structure of Calcium Phosphate Nanoparticles Synthesized with a Wet-Mechano-chemical Reaction. J. Phys. Chem. B 2002, 106, 5169–5176.
(87) Sandström, D. E.; Jarlbring, M.; Antzutkin, O. N.; Forsling, W. A. A Spectroscopic Study of Calcium Surface Sites and Adsorbed Iron Species at Aqueous Fluorapatite by Means of $^1$H and $^{31}$P MAS NMR. Langmuir 2006, 22, 11060–11064.
(88) Dietrich, E.; Oudadsess, H.; Le Floch, M.; Bureau, B.; Gloriant, T. In Vitro Chemical Reactivity of Doped Bioactive Glasses: An Original Approach by Solid-State NMR Spectroscopy. Adv. Eng. Mater. 2009, 11, B98–B105.
(89) Brunauer, S.; Emmett, P. H.; Teller, E. Adsorption of Gases in Multimolecular Layers. J. Am. Chem. Soc. 1938, 60, 309–319.
(90) Gregg, S. J.; Sing, K. S. W. Adsorption, Surface Area, and Porosity; Academic Press London: New York, 1982.
(91) Barrett, E. P.; Joyner, L. G.; Halenda, P. P. The Determination of Pore Volume and Area Distributions in Porous Substances. I.

The Journal of Physical Chemistry C
Computations from Nitrogen Isotherms. *J. Am. Chem. Soc.* **1951**, *73*, 373–380.