# Solid State $^{31}$P MAS NMR Detection of Hydrogen-bonded Phosphate Polymer in Calcium–Phosphate Composites

Akira Onoda, Mototsugu Doi, Kazuyuki Takahashi, Taka-aki Okamura, Hitoshi Yamamoto, and Norikazu Ueyama*
*Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043*

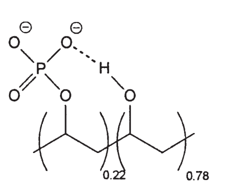
(Received November 26, 2003; CL-031149)

A novel poly(vinyl phosphate) ligand was synthesized as a model of biopolymer ligands containing the OH···O hydrogen bonds and $CaHPO_4{\cdot}2H_2O$ (brushite) composites with the polymer ligands were also prepared. Successful detection of the polymer ligand binding to the $CaHPO_4{\cdot}2H_2O$ crystals is achieved by $^{31}$P MAS NMR with 1 pda (single pulse with decoupling and phase cycling) sequence, which enhances $^{31}$P signals of the phosphate polymer having shorter $T_1$ value.

Organic template-directed mineralization enables crystallization of sophisticated inorganic architecture as biominerals in nature and it is basically important to develop a method for observation of such template molecules. On the edge of the biomineral Ca(II) phosphate crystals are supposed to be bound strongly with biopolymer ligands,[1,2] which are generally highly acidic and often contain phosphorylated amino acids,[3] such as dentin phosphoryn.[2] These proteins correspond to the biomineralization of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ and regulate the growing of the specific face of the crystals.[4,5] Statherin also contains similar acidic amino acid fragment of –Asp–Ser–Ser–, whose Ser residues are phosphorylated. Drobny et al. have performed solid-state NMR experiments using enriched samples of statherin and revealed that acidic region actually binds to hydroxyapatite crystals.[6] They have shown that the solid-state $^{13}$C NMR is powerful to observe organic molecules bound to inorganic crystals.

In the previous studies, we have applied $^{13}$C CP/MAS experiments to detect synthetic polymer templates in $CaCO_3$ composites.[7,8] We have synthesized a series of poly(carboxylate) ligands with strategically-oriented amide groups. These hydrogen-bonded poly(carboxylate) bind strongly to $CaCO_3$ crystals. $^{13}$C CP/MAS experiments of the $CaCO_3$ composites clearly show that the existence of the polymers which bind to Ca ion as carboxylate anion.

Here, we will show the successful observation of synthetic phosphate polymer ligand involved in $CaHPO_4{\cdot}2H_2O$ (brushite) composites using $^{13}$C and $^{31}$P MAS NMR experiments. Strong binding ability is essential for such analyses. We have already shown that NH···O hydrogen bonds to phosphate groups participate in strong binding with Ca ion.[9] Thus, we designed novel poly(vinyl phosphate) ligand containing the OH···O hydrogen bonds (Scheme 1) and synthesized hybrid composites of $CaHPO_4{\cdot}2H_2O$ crystal, which has similar Ca and phosphate array to that of hydroxyapatite. Our experiments have revealed that the NMR signals of template molecules involved in inorganic crystals can be detected with higher sensitivity using difference of $T_1$ values of polymers and inorganic phase.



**Scheme 1.** Phosphorylated poly(vinyl alcohol).

Phosphorylated poly(vinyl alcohol) **1** was synthesized by coupling reaction of poly(vinyl alcohol) and $H_3PO_4$.[10] $^{31}$P NMR experiment showed that the coupling reaction was performed successfully and about 22% of –OH groups were found to be phosphorylated from an elemental analysis.

Crystalline $CaHPO_4{\cdot}2H_2O$ was prepared with $(NH_4)_2$-$(HPO_4)$ and $CaCl_2$ solution in the presence and absence of the phosphate polymer.[11] The crystalline $CaHPO_4{\cdot}2H_2O$ was filtered and washed with excess water to remove the contaminated polymer ligand that is not involve in the crystals. Precipitation of $CaHPO_4{\cdot}2H_2O$ was determined by IR measurements. Figure 1 represents scanning electron micrographs of $CaHPO_4{\cdot}2H_2O$, $CaHPO_4{\cdot}2H_2O$ with poly(vinyl alcohol) and $CaHPO_4{\cdot}2H_2O$ with the phosphate polymer, **1**. $CaHPO_4{\cdot}2H_2O$ crystallized without polymer shows relatively large crystals which is larger than 20 µm. $CaHPO_4{\cdot}2H_2O$ crystal with poly(vinyl alcohol) has smaller size, ca. 10 µm and in the case of phosphate polymer, **1**, the composites are much smaller crystals of 2–5 µm size. The –OH groups in vinyl alcohol can form OH···O–P hydrogen bonds with inorganic phosphates. This intermolecular interactions is thought to inhibit the coordination of P–O oxygen atoms to Ca(II) ions. In the presence of **1**, the phosphate groups of the polymers can coordinate to Ca(II) ions. Thus, the SEM results clearly indicate that the polymer **1** strongly inhibits the crystal growth of $CaHPO_4{\cdot}2H_2O$ to give small crystals.

$^{13}$C NMR spectroscopy in the solid state was performed to detect the existence of the phosphate polymer in the $CaHPO_4{\cdot}2H_2O$ crystals. $^{13}$C and $^{31}$P NMR in the solid state was recorded on a Chemmagnetics CMX-300 with 4 or 5 mm$\phi$ pencil rotor cell. The measurement was performed using vacpX-tppm. Figure 2 shows the $^{13}$C NMR spectra of **1** and $CaHPO_4{\cdot}2H_2O$

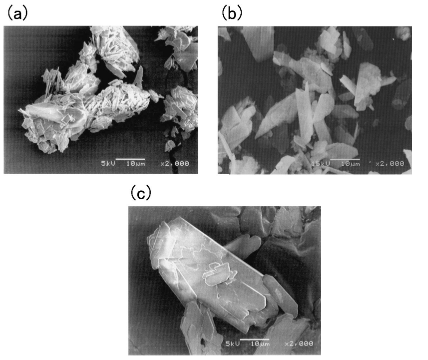


**Figure 1.** Scanning electron micrographs of $CaHPO_4{\cdot}2H_2O$ crystals (a) with 1% **1** polymer, (b) with 1% poly(vinyl alcohol) and (c) in the absence of the polymers.

Copyright © 2004 The Chemical Society of Japan

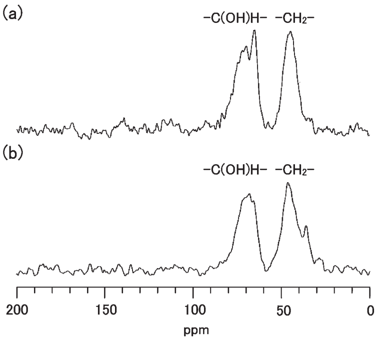


**Figure 2.** Solid-state $^{13}$C CP/MAS NMR spectra of (a) polymer **1** and (b) $CaHPO_4{\cdot}2H_2O$ crystals with the polymer.

crystals with **1**. The resonance originated from $–CH_2–$ and –CH(OH)– of the polymer main chain was observed. The similar signals are also detected in the $CaHPO_4{\cdot}2H_2O$ composite. Thus, it is suggested that the phosphate polymer, **1**, is actually involved in the crystals.

We have also employed $^{31}$P-{$^{1}$H} CP/MAS or simple MAS methods to detect $^{31}$P signals of polymers. Figure 3 shows the $^{31}$P NMR spectra of $CaHPO_4{\cdot}2H_2O$ and that with phosphate polymer. An observed sharp signal at 2.8 ppm is attributed to the one for the $HPO_4{}^{2-}$ ions (Figure 3a). In the case of $CaHPO_4{\cdot}2H_2O$ with 1% phosphate polymer, **1**, the signals of polymer was not sufficiently observed. Thus we have performed CP/MAS experiment with $CaHPO_4{\cdot}2H_2O$ with 10% phosphate polymer. $^{31}$P signals of side chain phosphate groups of the polymer are observed as broad signals at the side of $HPO_4{}^{2-}$ signal using vacpX (variable amplitude cross polarization) (Figure 3b). While using a 1 pda (single pulse with decoupling and phase cycling) sequence, these broad signals were observed with extensively high intensity at 1.1 and 4.5 ppm (Figure 3c). As a reference, we have synthesized the phosphate monoanion **2**, and the Ca(II) complex **3** of the polymer **1**. The $^{31}$P signals of **1**, **2**, and **3** are 1.1, 3.8, and 2.6 ppm, respectively. The Ca(II) complex, **3**, has an 1.5 ppm shifted resonance. The monoanion has a more shifted value (+2.7 ppm). The resonance at 1.1 ppm in $CaHPO_4{\cdot}2H_2O$ composite is supposed to be derived from the $^{31}$P signal in the phosphoric acid state. The 4.4 ppm signal comes from the phosphate anion which is thought to coordinate to Ca(II) ions of $CaHPO_4{\cdot}2H_2O$. The down field shift, 1.8 ppm, from Ca(II) complex **3** presumably indicates that the phosphate polymers bind in the dianion state. We have successfully identify the $^{31}$P signals of the phosphate polymer that is coordinating to Ca(II) ions on the surface of $CaHPO_4{\cdot}2H_2O$.

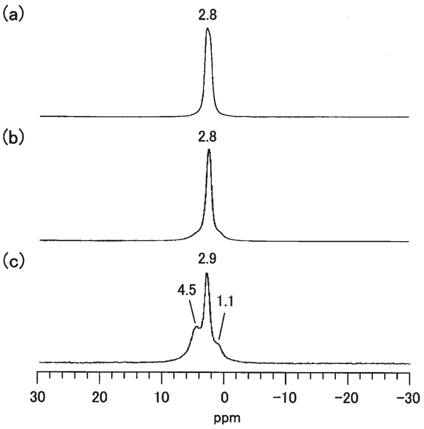


**Figure 3.** Solid $^{31}$P NMR spectra of (a) $CaHPO_4{\cdot}2H_2O$ crystals, (b) $CaHPO_4{\cdot}2H_2O$ crystals crystallized with 10% performed by vacpX sequence and (c) $CaHPO_4{\cdot}2H_2O$ crystals crystallized with 10% performed by 1 pda sequence.

The reason why we observe the polymer signal is utilizing difference of $^{31}$P $T_1$ values between the phosphate polymer **1** and $CaHPO_4{\cdot}2H_2O$ crystals. $^{31}$P $T_1$ values of the phosphate polymer **1** and $CaHPO_4{\cdot}2H_2O$ crystals are 50 and 170 s, respectively. Therefore, the $^{31}$P signals of the polymers have selectively observed as enhanced one. CP is very useful in observing the $^{13}$C signal of organic molecules in $CaCO_3$.[7,8] However, $CaHPO_4{\cdot}2H_2O$ crystals contain abundant protons which strongly enhance the $^{31}$P signals of inorganic phospahte. Thus, CP experiment is not applicable to the $CaHPO_4{\cdot}2H_2O$ composites. In conclusion, we have shown that the binding of phosphate polymer to $CaHPO_4{\cdot}2H_2O$ crystal can be detected by solid-state $^{31}$P NMR spectroscopy using 1 pda sequence, which enhances $^{31}$P signals with shorter $T_1$ values.

Support of this work by JSPS Fellowships [for A. O.; grant 2306(1999–2002)] and a Grant-in-Aid for Scientific Research on Priority Areas (No 10146103) from the Ministry of Education, Culture, Sports, Science and Technology of Japan is gratefully acknowledged.

### References and Notes

1 R. B. Frankel and S. Mann, in "ENCYLCOPEDIA of Inorganic Chemistry," ed. by B. R. King, John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapore (1994), p 269.

2 W. T. Butler, H. H. Ritchie, and A. L. J. J. Bronckers, "Dent. Enamel, 1997, Ciba Found. Symp.," (1997), Vol. 205, p 107.

3 H. Lowenstam and S. Weiner, "On Biomineralization," Oxford University Press, New York (1989).

4 H. Fueredi-Milhofer, J. Moradian-Oldak, S. Weiner, A. Veis, K. P. Minits, and L. Addadi, *Connect. Tissue Res.*, **30**, 251 (1994).

5 L. Addadi, J. Moradian-Oldak, H. Fueredi-Milhofer, S. Weiner, and A. Veis, "International Congress Series," (1992), Vol. 1002, p 153.

6 J. R. Long, W. J. Shaw, P. S. Stayton, and G. P. Drobny, *Biochemistry*, **40**, 15451 (2001).

7 N. Ueyama, T. Hosoi, Y. Yamada, M. Doi, T. Okamura, and A. Nakamura, *Macromolecules*, **31**, 7119 (1998).

8 N. Ueyama, H. Kozuki, K. Takahashi, A. Onoda, T. Okamura, and H. Yamamoto, *Macromolecules*, **34**, 2607 (2001).

9 A. Onoda, Y. Yamada, T. Okamura, H. Yamamoto, and N. Ueyama, *Inorg. Chem.*, **23**, 6038 (2002).

10 **Preparation 1**: 82% saponified poly(vinyl alcohol) synthesized from polyvinylacetate, POVAL417 (Kuraray Co Ltd.) Polyvinylalcohol (530 mg 12 mmol) was dissolved in 100 mL of DMF at 60 °C. DMF solution with $H_3PO_4$ (1.2 g, 12 mmol) and $N(n\text{-}Bu)_3$ (4.46 g, 0.12 mmol) was added and mixed solution was refluxed for 12 h. Excess ether was added and precipitated polymer was filtered. The polymer was stirred in 1 N NaOH solution and by acidifying the solution, the polymer was precipitated. The polymer was again dissolved in EtOH and HCl aq was added. Precipitated phosphoric acid polymer was dried in vacuo. Anal. Calcd for $C_{2n}H_{4.22n}O_{1.66n}P_{0.22n}{\cdot}(H_2O)_{0.1n}$: C, 37.86; H, 7.02. Found: C, 37.52; H, 7.07. **2**: 152 mg (5.4 mmol phosphoric acid unit) of **1** and 141 mg (5.4 mmol) of $(NEt_4)(OAc)$ was dissolved in MeOH and the solution was concentrated. Anal. Calcd for $C_{3.76n}H_{8.40n}N_{0.22n}O_{1.66n}P_{0.22n}{\cdot}(H_2O)_{0.30n}$: C, 47.30; H, 9.50; N, 3.23. Found: C, 47.07; H, 9.51; N, 3.28. **3**: 143 mg (5.1 mmol phosphoric acid unit) of **1** and 30 mg (5.1 mmol) of $Ca(OAc)_2$ were dissolved in water and the solution was concentrated. Evaporation of solvent was performed for several times. Anal. Calcd for $C_{2n}H_{4n}Ca_{0.11n}O_{1.66n}P_{0.22n}{\cdot}(H_2O)_{0.60n}$: C, 31.34; H, 6.84. Found: C, 31.40; H, 6.02.

11 **Crystaline $CaHPO_4{\cdot}2H_2O$**: $CaHPO_4{\cdot}2H_2O$ was synthesized by adding 5 mL of 1M $(NH_4)_2(HPO_4)$ to 5 mL of 1 M $CaCl_2$ solution in the presence of the phosphate polymer. The molar ratio of Ca(II) ion to phosphate dihydrogen group for **1** is 100. The suspended solution was stored overnight. The crystaline $CaHPO_4{\cdot}2H_2O$ was filtered and washed with excess water to remove the contaminated polymer ligand that is not involve in the crystals.

Published on the web (Advance View) March 23, 2004; DOI 10.1246/cl.2004.466