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固態核磁共振對多肽與生物活性玻璃相互作用之研究(1/2)

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計畫主持人：陳振中

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摘要

此計畫之重點是研究附於生物活性玻璃上的多肽之結構。在現階段，我們合成了一系列具有大表面積的矽玻璃，並成功導引羥基磷灰石在玻璃表面結晶，所合成的玻璃系統都通過了SEM, TGA與XRD等實驗的檢測。我們以固態核磁共振技術測量了磷酸鈣octacalcium phosphate，通過^{31}P雙量子與^{31}P-^{1}H HETCOR 實驗，我們確定了樣品中的PO_4^{3-}會局部轉化作HPO_4^{2-}。詳述此結果的論文已獲國際期刊 Journal of Solid-State Nuclear Magnetic Resonance接納。

Abstract

The main objective of this proposal is to study the conformation of a polypeptide when it is adsorbed to bioactive glasses. At the present stage, we have synthesized a series of sol-gel silica glasses with large surface area and successfully induced the formation of hydroxyapatite on the glass surface. The glass samples are well characterized by SEM, TGA and XRD techniques. Concerning the study of model compounds, we have assigned the ^{31}P solid-state NMR spectrum of octacalcium phosphate by ^{31}P double quantum and HETCOR spectroscopy. Our data reveal that substantial amount of the PO_4^{3-} groups at the P2 and P4 sites have been transformed to HPO_4^{2-} in our octacalcium phosphate sample. The NMR study of octacalcium phosphate has resulted in a manuscript which has been accepted by the journal of Solid-State Nuclear Magnetic Resonance.

I. Introduction

This project focuses on the interaction between bioactive glasses and polypeptides. There are three specific aims, *viz.* (i) characterization of the crystallization process of hydroxyapatite (HAP) on gel-silica glass surface; (ii) development of new solid-state nuclear magnetic resonance (SSNMR) technique for the determination of backbone torsion angle ψ of polypeptides; (iii) determination of the secondary structure of polypeptide adsorbed on gel-silica glass surface.

In the past seven months (Nov 2003 to May 2004), we had managed to prepare and characterize the target sol-gel glass systems, which were then soaked in simulated body fluid to induce the formation of HAP. In addition, considerable efforts were made to develop solid-state NMR strategy to study our model compounds, *viz.* octacalcium phosphate (OCP) and HAP. Concerning the synthesis of our target peptide, we have initiated a collaboration with Dr. Steve S. F. Yu at the Chemistry Institute of Academia Sinica. The laboratory of Dr. Yu is well-equipped for peptide synthesis and the first batch of peptide sample is expected to be available in the fall of 2004.

In the following sections we will summarize the results obtained thus far. Firstly, the protocol for glass preparation and HAP induction is briefly described. Then, the SEM, TGA and XRD data will be presented to show that the target glass system has been obtained and the formation of HAP on our glass sample has been successfully induced. Our solid-state NMR study of OCP is a successful one and we will present an excerpt of our manuscript which has been accepted by the journal of Solid-State Nuclear Magnetic Resonance.

II. Sample Preparation

Octacalcium phosphate

Urea (99.5%) , sodium phosphate monobasic dehydrate ($H_2NaPO_4 \cdot 2H_2O$) (99%) and calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$) (99%) were used as received (Acros). 10 mmol $Ca(NO_3)_2 \cdot 4H_2O$, 10 mmol $H_2NaPO_4 \cdot 2H_2O$ and 20 mmol urea were dissolved in 400 ml doubly distilled water and then sealed in a polypropylene container. The aqueous solution was kept at 100 °C for 3 hrs. The precipitates thus obtained were filtered, washed and then dried at 60 °C for 1 day.

Sol-Gel Silica Glasses

We have prepared a series of sol-gel silica glasses with high surface areas. On those glass samples which have calcium ions (30 %) incorporated, we have successfully induced the formation of hydroxyapatite. The glasses containing 70 mol % SiO_2 and 30 mol % CaO were prepared by mixing 7.2 g of polyacrylic acid (PAA), 26 ml deionized water, 16.6 g calcium nitrate tetrahydrate and 42 ml of tetraethylorthosilicate (TEOS). One ml of 14 N nitric acid was added to catalyze the hydrolysis of TEOS. After shaking vigorously for 5 min, the sol was hermetically sealed and aged at 80 ˚C. Gelation was observed to occur within a few hours at this temperature. After aging for 20 hrs the silica gel was cut into small pieces and refluxed in 200 ml ethanol-water at 90 ˚C for 24 hrs. The gel was then sintered at 500 ˚C for two hrs.

Simulated Body Fluid

Simulated body fluid was prepared by dissolving 20 g of NaCl, 0.88 g of $NaHCO_3$, 0.56 g of KCl, 0.57 g of $K_2HPO_4 \cdot 3H_2O$, 0.764 g of $MgCl_2 \cdot 6H_2O$, 1.043 g of $CaCl_2$, and 0.178 g of Na_2SO_4 in 2.5 liter of deionized water. The solution was filtered and then buffered at pH 7.4 with 19.56 g tris(hydroxymethyl)aminomethane and 10.99 ml of 12 N hydrochloric acid at 37 ˚C. The gel glasses were sieved and 200 mg of the glass powder were soaked in 500 ml SBF at 37 ˚C for various periods.

III. Results

Characterization of Glass Samples

A typical SEM image of our glass sample is shown in Fig. 1. The glass structure is very porous and the pore size is about 2 μm in diameter. The surface area was determined to be 330 m^2/g based on the B.E.T. nitrogen adsorption/desorption measurement. The amorphous state of the glass sample has been confirmed by XRD measurement. Thermogravimetric analysis has shown that the sintering temperature at 500 ˚C is high enough to remove the PAA residue in the glass sample. ^{29}Si NMR results are consistent to the literature data. As expected, the silicon species with four bridging oxygen (Q_4) constitute the

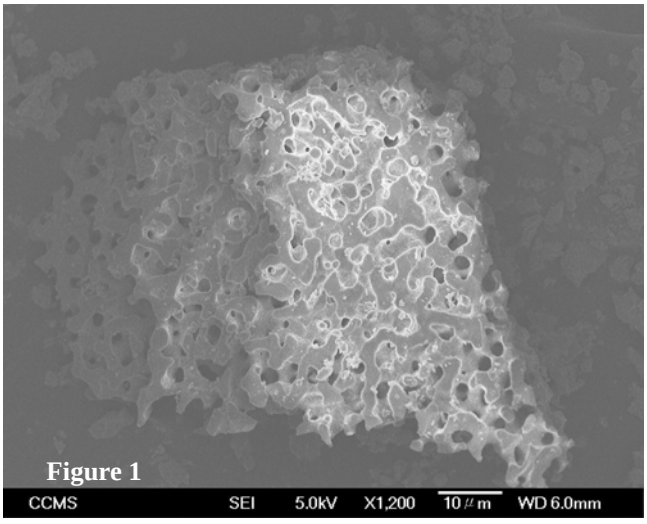

Figure 1

major ^{29}Si signal. After soaking in the simulated body fluid for 3 days, the SEM image of the glass has clearly shown the formation of HAP crystal (Fig. 2). The characteristic morphology of HAP is clearly seen in the image. Additional evidence for the HAP formation has been obtained by XRD measurement (Fig. 3). The HAP crystals formed on the glass surface have relatively poor crystallinity. ^{31}P solid-state NMR measurements are currently underway to investigate the average ^{31}P-^{31}P distance.

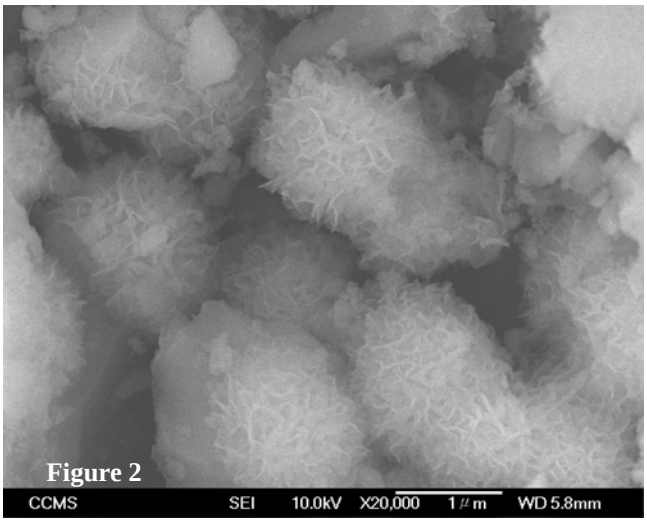

Figure 2

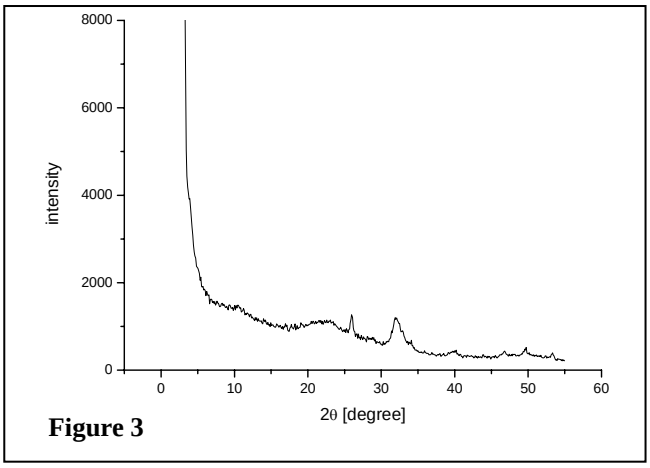

Figure 3

By varying the molar ratio of PAA and TEOS, we have prepared silica beads as shown in Fig. 4. The total surface area of these beads is similar to the gel glasses. In comparison the surface area of these beads are more accessible than that of the gel glasses. Therefore it would be of interest to study the interaction between peptide and these silica beads as well. Efforts are being made to induce HAP formation on these materials.

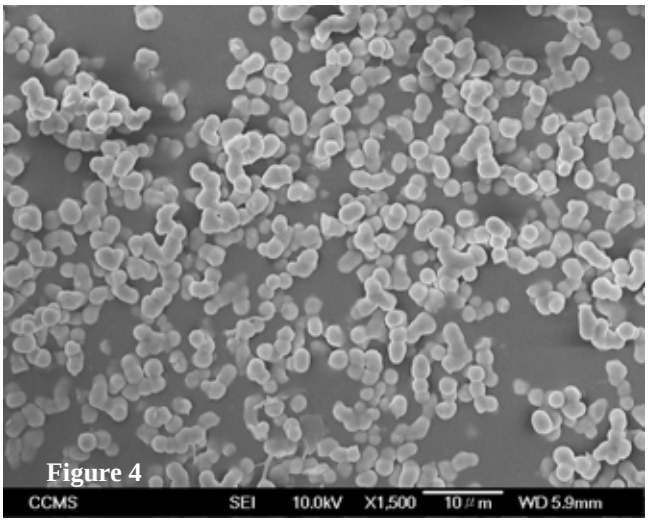

Figure 4

Model Compounds

In this project we have chosen OCP and HAP as our model compounds. These two compounds are structurally related and it has been postulated that OCP is the precursor phase of HAP in calcified tissue. In the literature the solid-state NMR study of OCP remains preliminary and no detailed spectral assignment of OCP NMR spectrum has been made. As such we have applied advanced techniques including ^{1}H{^{31}P} correlation and ^{31}P double-quantum spectroscopy to assign all the ^{31}P resonance peaks. As an illustration, we have reproduced the

$^{1}H\{^{31}P\}$ correlation spectrum in Fig. 5. The high-resolution ^{31}P spectrum of OCP has been assigned based on ^{31}P double-quantum and $^{31}P\{^{1}H\}$ HETCOR experiments. The ^{31}P peaks at -0.2, 2.0, 3.3 and 3.7 ppm are assigned to P5/P6, P3, P2/P4 and P1, respectively. This assignment is consistent with all the earlier works in the literature. Our data reveal that substantial amount of the PO_4^{3-} groups at the P2 and P4 sites have been transformed to HPO_4^{2-} in our OCP sample. Overall, our results have provided a useful basis for the identification of OCP as well as the study of OCP to HAP transformation.

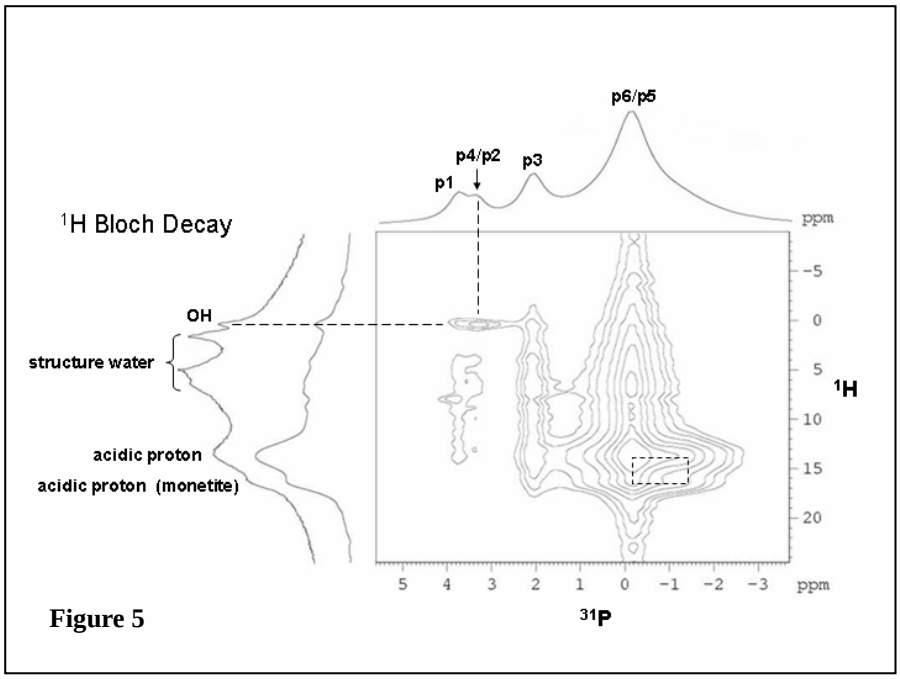

Figure 5

III. Self Evaluation

At the present time we have only one student (M. Sc.) in our research group. Nevertheless, we have obtained substantial progress in glass fabrication and solid-state NMR study of model compounds. Certainly, the publication of our first manuscript provides a strong impetus for the student's work. Due to limiting manpower we have not obtained much progress concerning the solid-state NMR study of polypeptides. Fortunately, in the coming semester there will be another three students joining our group and we are convinced that all the specific goals listed in our proposal will be accomplished in the second year of this project. As an independent evaluation of our work, we reproduced the referee's comment of our manuscript in the Appendix.

Appendix

Dear Professor Chan,

I shall accept your manuscript for publication in SSNMR provided that you address the following points made by my referee:

This paper uses modern solid state NMR techniques to identify and examine the connectivity of phosphorus sites (both to other phosphorus and protons) in octacalcium phosphate (OCP). Although OCP has been extensively studied by solid state NMR, the quality of the DQ 31P and the 31P{1H} HETCOR are sufficient that they make this paper a useful addition to the literature. There is intense and probably increasing interest in this material because of its occurrence in biological systems and in tissue engineering. The information this paper provides as to the structural detail is certainly an important part of the development of solid state NMR to tackle the key question of the structural mechanisms of the conversion of OCP to hydroxyapatite. The paper was in general well written, with lots of useful experimental detail. Referencing was good, highlighting many of the key papers. The abstract was a bit brief but it captures the main points of the paper. There is certainly no doubt that Solid State NMR is the most appropriate journal for this paper and I recommend publication. I did pick up the odd grammatical/typographical problem that needs correction and list these below.

Minor points

1. p3 line 9, insert a to read "in a biological"
2. p4 penultimate line immature should be premature
3. Throughout e.g. p6, Sec. 2.2, line 10 and elsewhere Fig. 1.. of the two full stops at the end one should be omitted. (see also Sec. 3.2, line 1, line 5, p9, last line, etc.)
4. p8, line 6, rather than an excerpt, better part of
5. p8, penultimate line, insert The to read "The same"
6. p10, line 9, are should be is
7. p10, line 11 little should be small
8. p11, line 8 add an s to read methods

Yours sincerely,

Jacek Klinowski

Editor-in-Chief, SSNMR