Experimental Report

Proposal: **8-02-665** **Council:** 10/2012

Title: Comparative studies on the colloidal calcium phosphate structure

This proposal is a new proposal

Researh Area: Biology

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Samples: beta-casein/CaP nanoclusters
OPN1-149/CaP nanoclusters
Amorphous CaP

Instrument	Req. Days	All. Days	From	To
D16	4	4	23/05/2013	27/05/2013

Abstract:

Many biofluids are supersaturated with respect to hydroxyapatite (HA) by forming complexes between phosphopeptides and amorphous calcium phosphate (ACP). The purpose is to maintain the integrity of bones and teeth without mineralising the surrounding soft tissues. We propose here to use contrast variation techniques to carry out a comparative study to characterise the core phase of nanoclusters prepared with either bovine osteopontin peptide (OPN 1-149) or casein, taking advantage of the q-range available at the D16 instrument.

Comparative studies on the Colloidal Calcium Phosphate structure

This report (proposal 8-02-665) concerns a comparative study to investigate the core phase of calcium phosphate nanoclusters (CPNs), successfully prepared with either bovine osteopontin peptide (OPN 1-149) or casein. A 2θ range was explored to seek for signs of higher order structure in the core phase of the nanoclusters, where standard crystalline phases were used as control samples. It was hypothesized that the use of hydrated samples, the non destructive nature of neutrons and the sample environment flexibility on D16 are critical advantages for this exploratory study. The CPN core is assumed to be amorphous, but so far the most sensitive techniques explored have been applied on dehydrated samples.

Sample preparation and characterisation: Various phases of calcium phosphate were prepared, according to protocols reported in the literature[1] (ACP, DCPD, OCP and HAP). OPNmix was isolated from bovine milk. Two hydrated samples of OPNmix CPN were produced from solutions of 10ml of 30mg/ml OPNmix CPN using 41%D_2O (contrast match point of the peptide) or 100% D_2O. Both solutions were centrifuged (85000g, 16h), yielding a hydrated pellet. A dried (lyophilised) OPNmix CPN sample was also prepared from an H_2O solution for comparison. FTIR spectra were (see Figure 1) recorded prior to and after data collection.

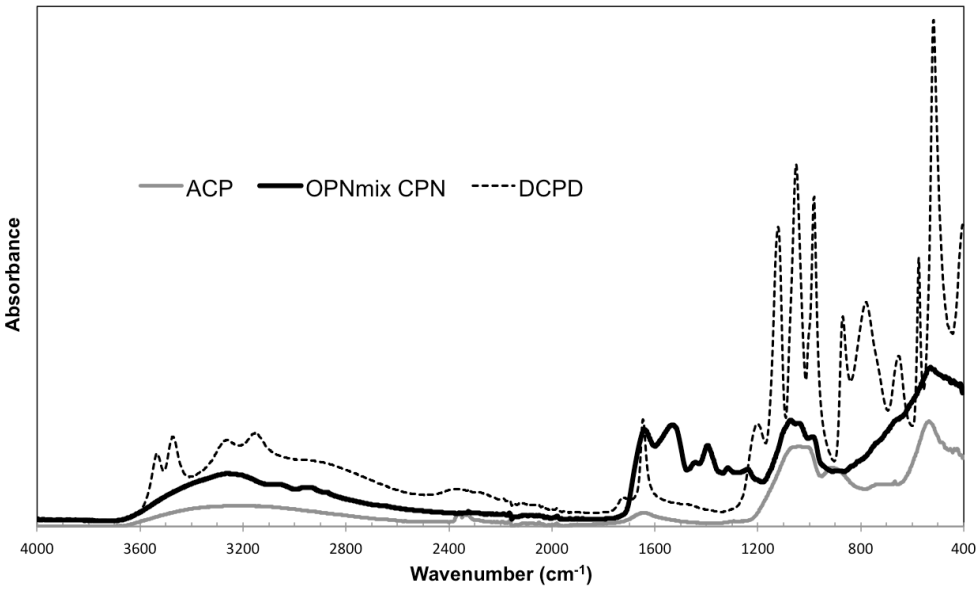
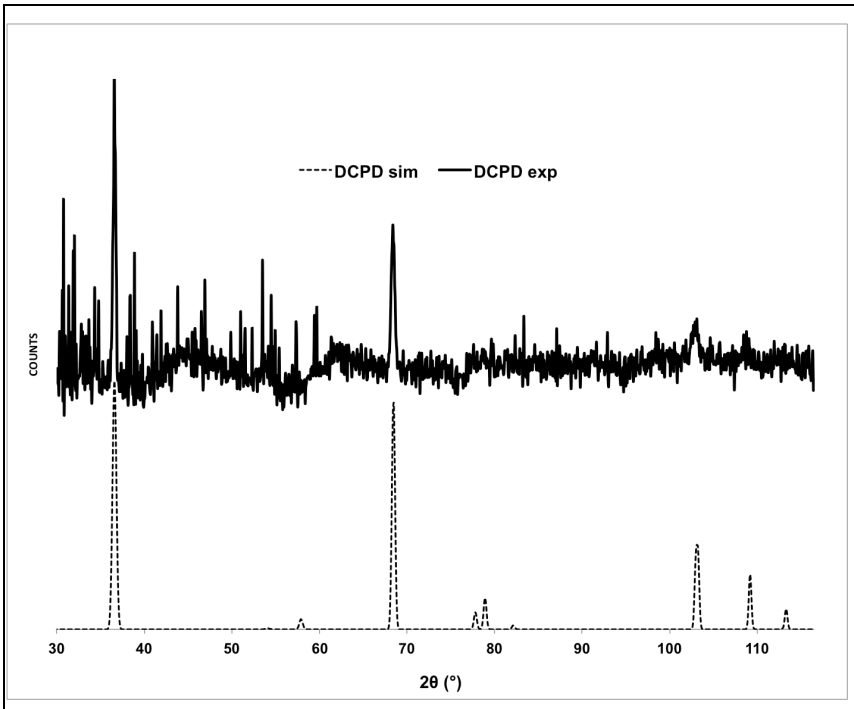


Figure 1 > FTIR spectra of dried samples: OPNmix CPN, DCPD and ACP (curves for the control samples are in close agreement with previously published results).

Formation of OPNmix-CPN was confirmed by dynamic light scattering measurements on a Malvern Zetasizer Nano S with a detection angle of 173 and a He-Ne laser (wavelength of 633nm). All light scattering measurements were recorded at 25 °C, with 3 repeat measurements of each sample for reproducibility. After 14 days the formed OPNmix-CPN had a hydrodynamic radius of 22.5 nm in solution (H_2O, 41% D_2O and 100% D_2O), in close agreement with previously collected data[2]. Pronase digested casein micelle samples were prepared using protocols previously described[3]. The hydrated micellar calcium phosphate was high speed pelleted to obtain a concentrated sample (85 000g for 16 hours at 8°C). Light scattering measured both before and after pelleting (ultracentrifuged samples were re-suspended in the same volume of buffer that they were initially equilibrated in) showed no significant changes.

Complementary X-ray diffraction data were also collected at room temperature (after neutron data collection) using a GeniX Cu high flux generator (1.5418Å) and recorded on a Mar345 detector with exposure times of 10mins per sample. All samples were mounted in a glass capillary of 0.5mm diameter (beam size at focus ~200x200 μm^2 with a divergence of 5mrad). X-ray data on both dried and hydrated samples provided data at high angles, where the D16 instrument setup limited the accessible angular range (relevant in the case of very small crystallites and/or heterogeneous order, where poor signal could mean that HAP diffraction can only be detected for stronger peaks at higher angles).

Data collection and results: Neutron scattering was measured at room temperature for all samples using a wavelength of 4.767Å ($\Delta\lambda/\lambda$=1%) and a 2θ scan of 5-115°. A beam diameter of 3mm (calculated divergence 0.33˚ directly from an image of the attenuated beam) and an average flux on the sample of $1x10^6$ neutrons.$cm^{-2}s^{-1}$ was used.



All samples were deposited in spherically symmetric sample containers (5mm diameter) and their contribution was subtracted from the sample data. Exposure times of 30mins per sample (exposures of up to 2h were tested but did not improve the signal to noise ratio) were used. Data reduction was carried out using LAMP (see Figures 2 and 3). Complementary X-ray diffraction data were also collected at room temperature using a GeniX Cu high flux generator (~400x10^6photons.s^{-1}) with exposure times of 10mins per sample. X-ray data in the range 25-1.9Å showed no higher order crystallinity for any of the CPN samples.

Figure 2 < Neutron diffraction pattern of the DCPD sample: a sharp Bragg peak was observed for at a 2θ of 36.3° (I/σ)=20.9) and a broader peak at 67.8° (I/σ=16.8), matching the simulated pattern as shown (simulation based on the previously published crystal structure of DCPD).

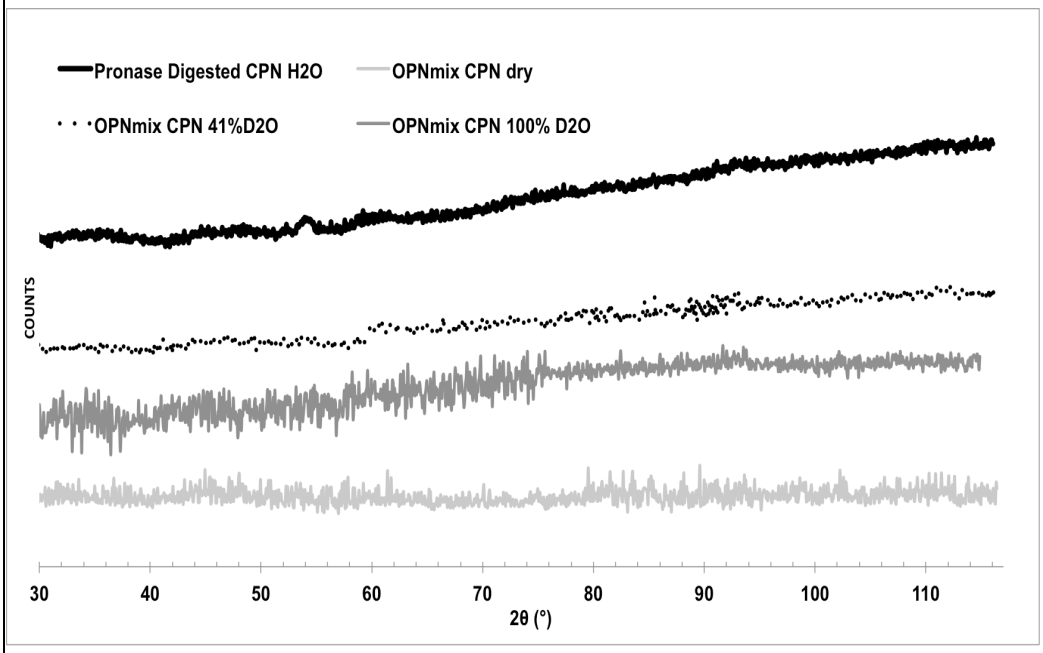


Figure 3 > The plot shows an overlay of the neutron diffraction patterns of the OPNmix and pronase digested CPN samples. Intensities are arbitrary. The patterns are similar to ACP, where no unambiguously identifiable diffraction rings are detected.

Discussion: Hydration is central to the formation and stability of all calcium phosphate phases. Hydrated ACP evolves into higher order forms through a process of solution and redeposition. Maturation kinetics can therefore be affected by sample preparation protocols (by drying, chemical or kinetic stabilisation). It was shown for example that in vitro ACP can require at least 22h for maturation into more crystalline phases and as long as three weeks. The type and size of potential crystallites in CPN under biologically relevant conditions is particularly difficult to assess given experimental limitations in terms of sensitivity and time scales. Assuming isotropic distribution of crystallites and ignoring additional strain effects, the size L of possible crystallites present in the CPN core can be approximately estimated using Scherrer's formula (eq.1).

$$L = \frac{K\,\lambda}{\beta \cos\theta} \quad \text{(eq. 1)} \qquad \text{which can be re-written as:} \qquad L = \frac{2\pi K}{\Delta q} \quad \text{(eq. 2)}$$

β in equation 1 is the breath of the diffraction peak (Δ2θ), and K is a constant related to crystallite shape (typically 0.62-2.08, arbitrarily taken here as 0.94 corresponding to spherical cubic crystals). Equations 1 and 2 can be combined for a Δ2θ range, where the average number of Bragg planes contributing to a certain hkl reflection is related to d spacing by:

$$N_{hkl} = \frac{L}{d_{hkl}} \qquad \text{(eq. 3)}$$

Table I shows the estimated crystallite sizes using equation 3 at varying peak widths (only peak breath values larger than the instrumental broadening were considered) and Bragg angles. The average number of Bragg planes was calculated using the most unfavorable case of the smallest possible known unit cell edge (0.58nm) for calcium phosphate, corresponding to the monoclinic unit cell of DCPD (a = 0.58 nm, b =1.52 nm, c =0.0.62nm, β= 116°).

Table I. Minimum estimated crystallite sizes of calcium phosphate at the D16 instrument for an incident neutron wavelength of 4.767Å and four values of the diffraction peak breath β.

β (°)	2θ (°)	L (nm)	N_{hkl}
1	5	25.7	44
	115	29.3	50
2	5	12.8	22
	115	14.6	25
4	5	6.4	11
	115	7.3	12
8	5	3.2	5
	115	3.7	6

Relatively long neutron wavelengths with appropriate coherence lengths allow for small crystallites to be probed, while the use of heavy water can be deployed to enhance contrast in neutron data and assist in the FTIR peak assignment. This can be a key advantage in complex systems where diffraction peaks can overlap, making the interpretation of X-ray diffraction ambiguous in the absence of a correct model.

For the CPN systems explored in this particular study, no crystalline order was detected, even for the significantly larger core of the CPN formed with OPNmix. This does not completely rule it out: effects of crystallite size, number, homogeneity and density are correlated and add a complexity to the diffraction pattern that can ultimately determine the sensitivity of the experiment. It is unlikely that crystallites smaller than 3.2nm can be identified by neutron diffraction at the D16 instrument, in its 2013 configuration (H53) and wavelength. If the casein CPN core radius is taken as reference (4.8nm diameter), the experimental resolution at the instrument can limit the size of crystallites detectable. The most unfavorable, arguably most likely presence of the monoclinic crystal form of DCPD could yield neutron diffraction peak breaths at the D16 instrument of 8˚ or higher. For OPNmix-CPN, the 35nm diameter of the core allows however for larger crystallites and higher confidence in the ability to unambiguously identify peaks in the data recorded.

Apart from the clear interest of detailed structural information on casein and OPN-CPN[4-5], experimental data can establish useful guidelines and references for future studies on these, or other complex systems (where the effects of sample concentration and compaction, for example, should be systematically characterised; for this experiment problems with the detector electronics prevented further tests to be carried out). The results described in this report are included in a manuscript currently under preparation for publication.

References:

[1] V. Dorozhkin, S. (2012). Int. J. Materials and Chemistry 2, 19–46.

[2] C. Holt, S. Lenton, T. Nylander, E. Sørensen, S. Teixeira (2013). J. Struct. Biol., doi: 10.1016/j.jsb.2013.11.009.

[3] Holt, C., Davies, D. T. & Law, A. J. R. (1986). J. Dairy Research 53, 557–572.

[4] C.Holt, J. Carver, H. Ecroyd, D. Thorn (2013), J. Dairy Sci. 96(10):6127-46.