

In vitro and in vivo study of commercial calcium phosphate cement HydroSet™

Niall Kent¹, Gordon Blunn², Natalia Karpukhina¹, Graham Davis¹, Roberta Ferro de Godoy², Rory Wilson³, Melanie Coathup², Lyris Onwordi², Wenyu Quak², Robert Hill¹

¹ *Dental Physical Sciences, Institute of Dentistry, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Mile End Road, London E1 4NS, United Kingdom.*

² *John Scales Centre for Biomedical Engineering, Institute of Orthopaedics and Musculoskeletal Science, University College London, Royal National Orthopaedic Hospital, Stanmore, United Kingdom.*

³ *School of Engineering and Materials Science, Queen Mary University of London, Mile End Road, London E1 4NS, United Kingdom.*

Corresponding Author: Niall Kent

Email: n.w.kent@qmul.ac.uk

Abstract:

The commercial calcium phosphate cement, HydroSet™, was investigated *in vitro*, studying phase formation, compressive strength and setting time, followed by an ovine *in vivo* study to measure osseointegration, bone apposition and bone-to-graft contact. The X-ray diffraction and ³¹P MAS NMR results showed the initial formation of octacalcium phosphate and hydroxyapatite at one hour. Over seven days the octacalcium phosphate transformed to apatite, which was the only constituent of the cement at 28 days. This apatite phase is thought to be a calcium deficient apatite. In the scanning electron microscopy, histological images of twelve-week ovine *in vivo* results showed a high degree of osseointegration, 92.5 %. Compressive strength comparisons between *in vitro* and *in vivo* measurements showed a dramatic difference between the *in vitro* measurements (highest 25.4 MPa) and *in vivo* (95 MPa), attributed to bone ingrowth into the cement *in vivo*. To the best of our knowledge this is the first time phase evolution of HydroSet™ and the properties studied *in vitro* complement the *in vivo* evaluation of the cement in publication. Significance of a new finding of initial formation of octacalcium phosphate in this cement is discussed.

Keywords: Calcium Phosphate Cement, HydroSet™, Octacalcium Phosphate, Hydroxyapatite, In vivo, In vitro

Introduction

Calcium Phosphate Cements, CPCs are calcium phosphate based synthetic bone graft materials, which form as a result of the reactions between calcium phosphate salts^{1, 2}. On mixing with water the salts react to form apatite³, the mineral phase of bone and tooth^{4, 5}. CPCs have uses in both orthopaedics and dentistry where they are used as bone substitute materials to replace missing or diseased bone⁶. They are advantageous because of their injectability, which allows the surgeon to inject the setting cement paste into the implantation site minimally invasively^{7, 8}. These materials are also *in vivo* setting which allows them to mould to the shape of the implantation site once implanted, which ensures complete void filling^{9, 10}. Unlike CPCs, pre-set bone substitutes do not have this advantage and cannot set *in vivo*.

The first commercial calcium phosphate cement was developed by Chow in 1983². In the first patent on calcium phosphate cements tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) (or dicalcium phosphate di-hydrate (DCPD)) were used as the initial reagents. The authors discovered that leaving these mixtures in test tubes led to a 'hard' deposit after a few hours. These calcium phosphate salts are more soluble at neutral pH than hydroxyapatite¹¹ and when mixed with water produce hydroxyapatite. As a consequence of this discovery various animal studies were conducted¹²⁻¹⁴ which demonstrated precipitation of nano-crystalline hydroxyapatite in these compositions. On implantation highly biocompatible HA was readily replaced by new bone¹⁴. This cement composition using TTCP and DCPA (or DCPD) was approved by the FDA in 1996 and became the first commercial calcium phosphate cement³.

These cements have varied medical applications and are specifically useful in orthopaedic surgery due to the compositional similarity to bone. They are implanted into bone in many procedures; they are used in craniofacial surgery, skeletal fractures, hip replacements, vertebroplasty, kyphoplasty and in other corrective and restorative orthopaedic procedures¹⁵⁻¹⁸.

Typical cement setting occurs via a dissolution/precipitation route¹, whereby the two calcium phosphate salts dissolve into the aqueous solution to their constituent Ca^{2+} and PO_4^{3-} ions. These ions then, precipitate to hydroxyapatite. HydroSet™ is an apatite based calcium phosphate cement consisting of a powder and solution, that are mixed together to initiate the setting reaction. The powder contains TTCP (73%) and DCPD (27%) and the solution H_2O , Na_2HPO_4 , NaH_2PO_4 and Polyvinylpyrrolidone (PVP). To the authors knowledge no previous studies have been published studying the *in vitro* phase evolution of HydroSet™. Whilst the combination TTCP and DCPD have been studied extensively HydroSet™ also contains polyvinylpyrrolidone in its formulation, which could potentially affect phase evolution. Hannink *et al.* studied the phase evolution *in vivo* and found the formation of hydroxyapatite within the cement; it was also found that some starting powder remained unreacted within the implant for up to twenty eight weeks¹⁹.

The aim of this study was to study the *in vitro* and *in vivo* properties of the commercial calcium phosphate cement HydroSet™ including the structural characterisation combining the XRD and ^{31}P MAS NMR. The latter allows probing the local environment of the phosphorus atoms in solids and delivers the detailed information regardless whether the environment is crystalline or amorphous. The ^{31}P MAS NMR has been shown to be specifically useful for the identification of octacalcium phosphate^{20, 21}. Tseng, et al. ²² showed previously the ability of ^{31}P MAS NMR to successfully differentiate between octacalcium phosphate and hydroxyapatite and even study the transition between the two. Another advantage of the ^{31}P MAS NMR probe is high sensitivity that allows the detection any

of phosphorus-containing species present in solid that are in small amounts. The *in vitro* aspect was to study phase and mechanical evolution over twenty-eight days of immersion in Tris buffer solution. Using an ovine femoral implant model for *in vivo* study we aimed to measure osseointegration, bone apposition and compressive strength. The ovine model was used because bone apposition rates better match those found in humans than the rat or rabbit model and the approximate size and weight is similar as to that in humans²³.

Materials & Methods

***In vitro* Preparation**

HydroSet™ was mixed according to the manufacturer's instructions to produce a paste. This cement paste was filled into cylindrical steel moulds with a height of 6 mm and diameter of 4 mm. The cylinders were 'over-filled' and sandwiched between two steel plates. The moulds and plates were then clamped together using a G clamp. The clamp was then placed into a 37°C oven (Borolabs incubator, Borolabs Basingstoke, UK) for 120 minutes. The clamp and plates were then removed and the cements, whilst in the moulds were ground with silicon-carbide paper to ensure a flat surface at each end of the cylinder.

Each cylinder was then immersed in 10 ml of Tris buffer solution, as prepared previously²⁴, (in 15 ml plastic centrifuge tubes) for 1 hour, 1 day, 7 days or 28 days. Eight cement cylinders were immersed at each time point for each cement composition for compressive strength measurements. The samples were stored in a 37°C oven for their respective time-points. After being stored each cylinder was removed and patted dry and the compressive strength measured immediately.

Cement Property Characterisation

Compressive Strength Measurements

Compressive strength was measured using an Instron 5567 mechanical property testing machine (Instron, High Wycombe, UK) using a 1kN load cell. Cylindrical specimen after their respective immersion periods were placed between two steel plates without drying with a 1 cm square damp filter paper above and below the specimen. Force was applied at a displacement rate of 1 mm/min. The test was stopped either automatically by the machine when fracture was detected or when it was evident by eye that fracture had occurred.

Setting Time Measurement

The setting time of each cement sample was measured using the Gilmore needle test according to the ISO standard ISO (9917-1:2007(E)). The HydroSet™ Cement pastes was prepared according to the manufacturer's instructions, the cement paste was filled into cylindrical moulds (Height 4 mm, Diameter 8 mm) using a spatula. To measure the initial setting times the 'lighter' Gilmore needle (2.12 mm dia. 113.4 g Wt.) was lowered onto the cement surface and left for 5 seconds, the setting time was taken until the needle tip no longer makes an indentation on the cement surface. To measure the final setting time the larger Gilmore needle (1.06 mm dia. 453.6 g Wt.) was used and again the needle was lowered onto the surface of the cement paste and left for 5 seconds, the final setting time was taken when the needle no longer makes an indentation on the cement surface.

Cement Phase Analysis

X-ray Powder Diffraction

Powder XRD experiments for the cements were run on X'Pert Pro PANalytical diffractometer with Bragg Brentano θ/θ geometry with Cu K α radiation operating at 45kV and 40mA. Data were collected

from 3 to 70 2θ° in increments of 0.0334°. Beam divergence was set to ¼°. The detector used was an X'Celerator RTMS detector and the data collection time at each point was equivalent to 200 seconds. The samples were mounted on zero background silicon single crystal substrates.

Magic Angle Spinning Nuclear Magnetic Resonance

The ³¹P MAS NMR experiments were run on Bruker 600MHz spectrometer at the 242.9MHz resonance frequency. The powder samples were packed into 4mm rotor and spun at 11-12kHz. The measurements were done using 60s recycle delay and 85% H₃PO₄ was used to reference the chemical shift scale.

Surgery and Test Material Administration

All relevant licenses and Ethics approval were in place and procedures carried out in compliance with the UK Home Office as stated in the Animal Scientific Procedures Act (1986). Prior to surgery each sheep was administered the pre-med RompunTM [xylazine] and anaesthesia was induced by intravenous administration of ketamine and midazolam and then maintained using isoflurane and oxygen. The anaesthetised animal was prepared for surgery and the medial femoral condyle was exposed by a medial approach. Two holes were drilled, one in a proximal position and one in a distal position with >4mm between the holes. The holes were drilled to a standard depth of 15 mm made with an 8.0 mm drill bit. 1 mm holes were drilled either side of the defect and 1 mm tantalum beads were inserted in order to correctly locate the defects on retrieval using radiography. After irrigating with sterile saline, the test material was pressed into place. The wound was closed and the contra-lateral medial femoral condyle exposed by a medial approach. In a similar manner two holes were drilled, irrigated with sterile saline, test materials inserted and the wound closed.

At the time of surgery, an antibiotic injection of CeporexTM [cephalexin] was given intramuscularly at a dose of 5mls per animal. Prior to recovery from anaesthesia all animals received an analgesic injection of VetergesicTM [2mls; 0.6mg Buprenorphine] administered as an intra-muscular injection. CeporexTM injections were also given postoperatively for 3 days. VetergesicTM was administered daily on the three successive days post-surgery in all animals.

Bone marking injections

The animals received their first bone marking injection on day 63 +/- 2 (9 weeks) followed by a second bone marking injection 14 days later. The first bone marking injection was an intravenous administration of Alamyacin 10 [oxytetracycline] at a dose of 15 mg/kg body weight and the other an intravenous administration of Calcein green at a dose of 10 mg/kg body weight.

Terminal procedures

The animals were sacrificed at 12 weeks post-surgery. All animals were euthanased by an overdose of pentobarbital. After sacrifice the femoral condyles including a small part of the femoral shaft were removed from the animal. Samples were radiographed and transverse cuts were made through each defect using a diamond band saw. This produced a small slab with an exposed transverse cut face of the implant which was surrounded by at least 5 mm of bone. One half of each sample was used for mechanical testing and immersed in phosphate buffered saline. Samples were compression tested on the day of sacrifice. Specimens for histology (and following mechanical testing) were immediately fixed in at least 4 times the tissue volume of 10% neutral buffered formal saline.

Laboratory phase

Histological processing and analysis

Each sample was dehydrated in serial dilutions of alcohol, followed by three changes in absolute alcohol before immersing in Chloroform to de-fat the specimens. Specimens were returned to absolute alcohol

before being immersed in a 50:50 solution of acrylic resin and absolute alcohol. Samples were transferred to concentrated acrylic resin solution prior to being polymerized in acrylic resin. After embedding, the implant and the bone were sectioned using a band saw. Sections were taken longitudinally through the centre of the implant in the coronal plane. Thin slices (100 - 60 μm) were then prepared using an EXAKT saw.

Bone remodelling rates adjacent to the defect surface and within the defects were determined in these thin sections (100 – 60 μm) before staining. This was achieved by measuring the distance between bone markers under a fluorescence microscope and using our image analysis system (Figure 1). In all animals, bone turnover rates were calculated from regions along the length of the defect and resulted in a minimum of 7 regions of interest being measured per slide. Measurements were taken using the $\times 5$ objective lens. Apposition rates were calculated in $\mu\text{m day}^{-1}$.

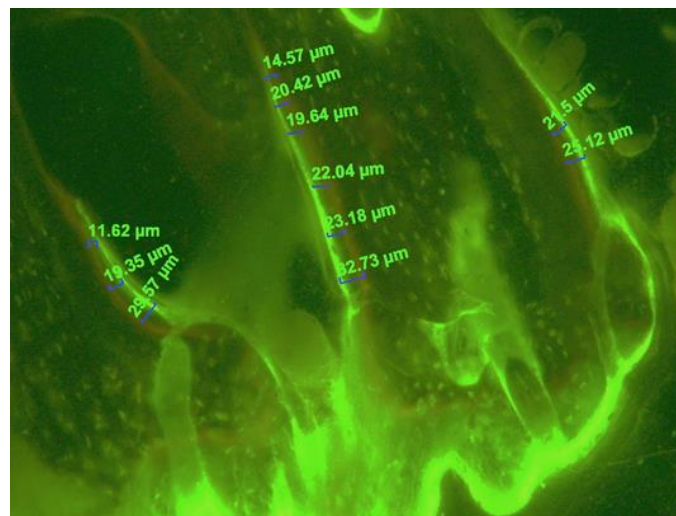


FIGURE 1: Fluorescence microscopy of specimen

Following fluorochrome marker measurement and analysis, thin sections were stained with Toluidine Blue for 15 minutes and Paragon for 10 minutes and the amount of new bone, bone-to-graft contact and test material present within the defect assessed using an image analysis system (Axiovision 4.5; Carl Zeiss, Imaging Associates Ltd, UK).

In addition to quantifying the measurable difference between the two bone markers, image analysis was also used to quantify the amount of bone-cement contact. This involved capturing images along the entire bone-cement interface using a $\times 10$ objective lens and a digital colour camera on the image analysis system. Assessments were used to evaluate the proportion of bone and soft tissue in two dimensions.

Backscattered Scanning Electron Microscopy.

Sections from each test group and at both time-points were sputter-coated and prepared for viewing using SEM (JEOL scanning electron microscope JEOL Welwyn Garden City, Hertfordshire, UK).

X-ray Microtomography

Mechanical Testing

The compressive strength of each of the cements was tested using an indentation method in two central positions (at least 2 mm apart) within the cement mantle. Testing was performed on a screw driven

Zwick Proline 500 test machine fitted with a 500 N load cell with an accuracy of 0.5 N. Compression testing was performed using a custom-made jig (Figure 2).

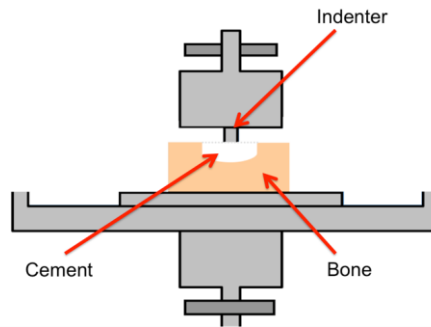


FIGURE 2: Test jig configuration for compression testing.

The indenter was cylindrical and measured 3 mm in diameter and was positioned in the centre of the test specimen. Before testing could begin, the upper crosshead platen was lowered so as to just make contact with the specimen. In practice this was achieved by applying a preload of 2 N. Load was applied axially to the specimens with a crosshead speed of 5 mm/min. Specimens were loaded up to yield and beyond into the collapse plateau region of behaviour, as defined by Gibson²⁵ for elastic-brittle foams. The compressive strength (in MPa) applied to the sample throughout the test was calculated using equation 1.1.

$$\text{Compressive Stress} = \frac{\text{Applied Load, in N}}{\pi (\text{radius of indenter, in mm})^2} \quad (1.1)$$

Results

In vitro Studies

The X-ray diffraction of the starting material in Figure 3 shows that the phases present are DCPD, tetracalcium phosphate and DCPA. The ³¹P MAS NMR of the starting material, the bottom spectrum in Figure 4, confirms this. At 1 hour in Tris buffer solution the XRD (Figure 5) shows the presence of DCPD, tetracalcium phosphate and octacalcium phosphate. The latter was identified on the X-ray diffraction from low intensity lines at 4.7 and 9.8 degrees two theta. The X-ray diffraction shows that in the 1 day, 7 day and 28 day samples two phases are present, tetracalcium phosphate and hydroxyapatite. The only apparent change is the relative intensities of the two phases; the tetracalcium phosphate decreases in relative intensity whereas the hydroxyapatite increases in its relative intensity.

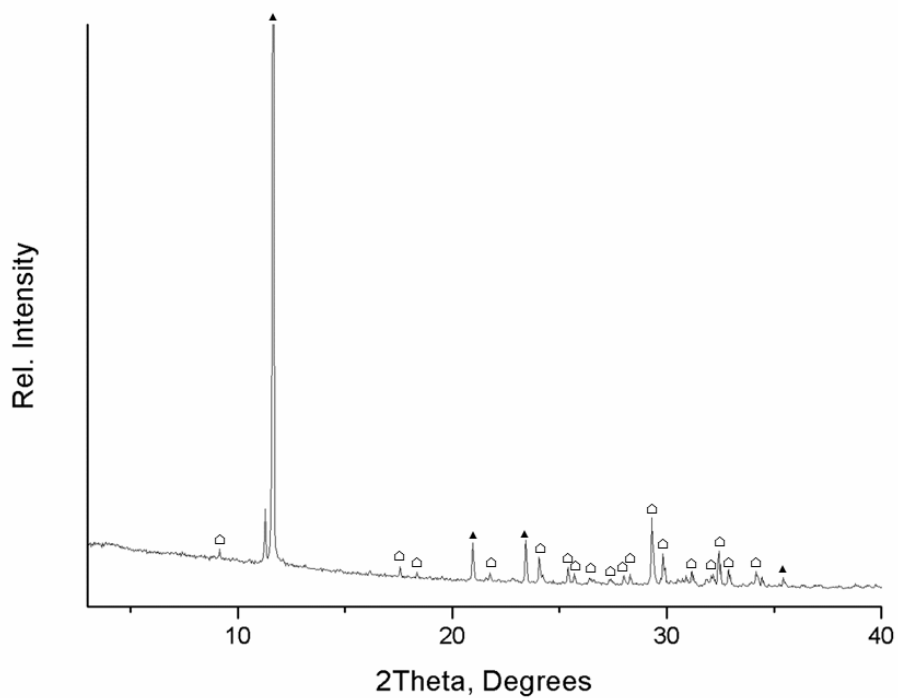


FIGURE 3: XRD of the starting powder.

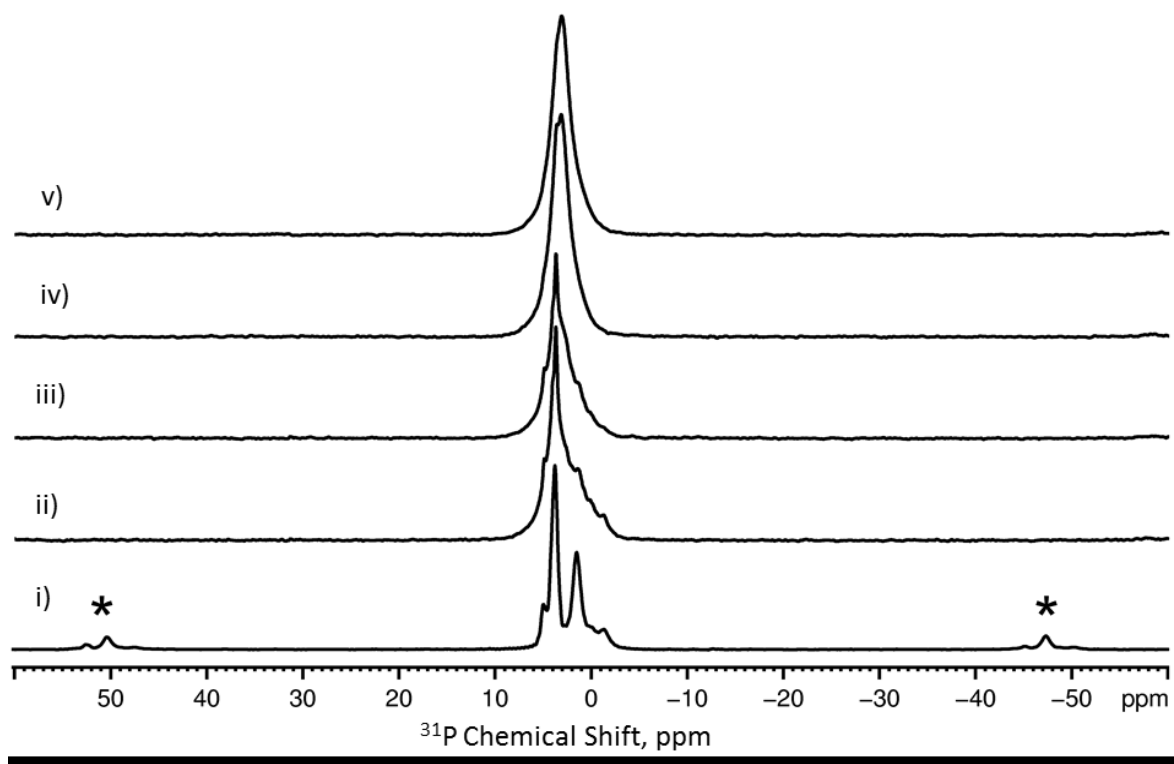


FIGURE 4: ^{31}P MAS NMR spectra for the starting powder (i) and cements matured for (ii) 1 hour, (iii) 1 day, (iv) 7 days and (v) 28 days of immersion in Tris buffer. The asterisks show spinning side bands.

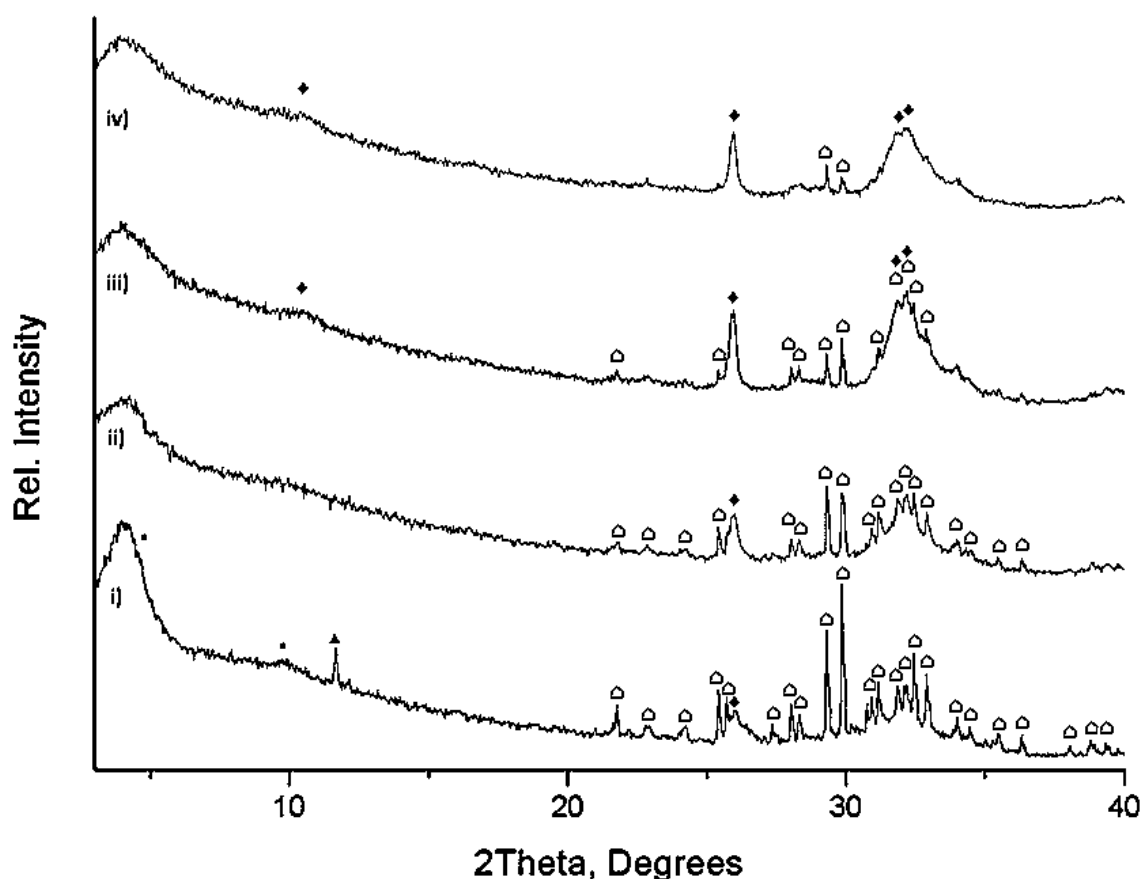


FIGURE 5: XRD patterns of the cements matured for (i) 1 hour, (ii) 1 day, (iii) 7 days and (iv) 28 days of immersion in Tris buffer. The open symbols are for tetracalcium phosphate; the triangles are for the DCPD; squares for the OCP and the diamonds for the apatite.

The ^{31}P MAS NMR results (Figure 4) show that at 1 hour tetracalcium phosphate is present with peaks at 3.8 and 5.0 ppm. There is a fairly broad shoulder at 2.8 ppm suggesting hydroxyapatite is present, as well as a peak at 1.4 ppm indicating a small fraction of DCPD. There are also several peaks at 3.6, 2.0 and -0.2 ppm showing that OCP is present. The ^{31}P MAS NMR results show tetracalcium phosphate is clearly still present up to 1 day of the immersion, as can be identified by the peaks at 3.8 and 4.8 ppm. This is true also for DCPD where a peak at 1.4 ppm is present. The peaks at -1.4 and -0.2 ppm show a small fraction of DCPA is still present in the cement phase from the starting materials. The DCPA was identified only on the ^{31}P MAS NMR and not on the X-ray diffraction by chemical shift at -1.4 and -0.2 ppm. The 7 day ^{31}P MAS NMR spectrum has a peak at 3.0 ppm of hydroxyapatite and two additional peaks at 3.7 and 4.9 ppm of tetracalcium phosphate. The 28 day sample has a main peak at 2.9 ppm (hydroxyapatite), a shoulder at 3.7 ppm and a very small shoulder at 4.9 ppm (tetracalcium phosphate).

Table 1 Compressive strength of HydroSet™ immersed in Tris buffer solution at 1 hour, 1 day, 7 days and 28 days.

Compressive Strength (MPa)			
1 Hour	1 Day	7 Days	28 Days
14.2 (± 1.4)	25.4 (± 2.2)	13.6 (± 1.8)	25.1 (± 3.5)

HydroSetTM compressive strength values ranged from 13.6 to 25.4 MPa as shown in Table 1. The initial setting time of the HydroSetTM sample was 6 min and the final setting time was 19 min.

In vivo Studies

Bone apposition rates were measured in bone adjacent to cements in all of the thin sections prepared in this study. The number of measurements made per slide varied according to the distribution of the two markers within the bone, however, a minimal number of 7 regions of interest were measured from each slide. Results showed rate of bone turnover measured was 1.191 (± 0.345) $\mu\text{m/day}$. Extensive % bone-cement contact was measured in all experimental groups the mean percentage contact of 92.5 %. Results following mechanical tests showed that the compressive strength of samples in each of the groups was similar at 95 MPa (Table 2). Figures 6, 7 and 8 show photographs and electron micrographs of the bone/cement interface showing high levels of new bone formation on the cement surface. Figure 9 shows an X-ray micrograph transection of the HydroSetTM within the implant site twelve weeks after surgery. The micrograph shows the relatively high radio-opaque HydroSetTM cement block integrated strongly with the trabeculae and disorganised newly formed bone surrounding the cement surface and adjacent trabeculae.

Table 2 The minimum, maximum and mean compressive strength values obtained in each of the groups.

Minimum (MPa)	Maximum (MPa)	Mean (MPa)	Std. Deviation
78.4	142.2	95.0	27.2

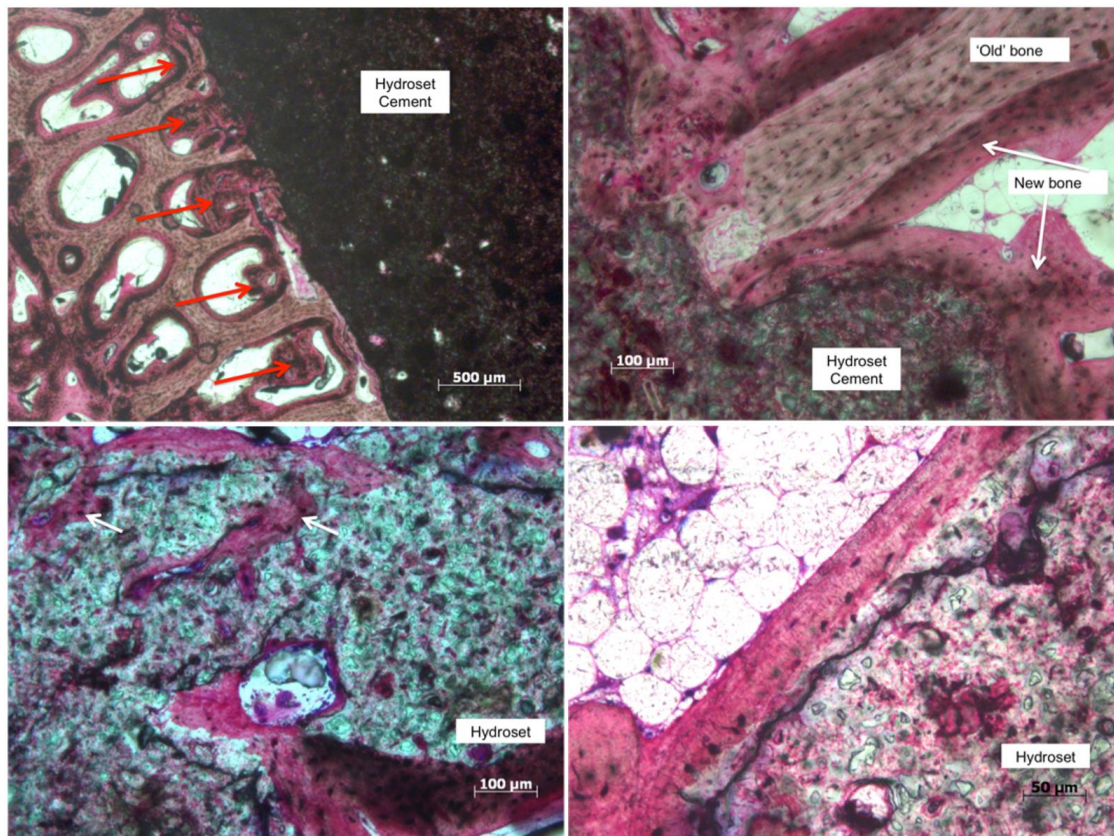


FIGURE 6: Photomicrographs of histological staining showing: Top Left) new bone formation to the bone-cement interface. The red arrows point to clusters of new bone formation within the older trabecular network. Top Right) New bone formation adjacent to the bone-HydroSet™ interface. Bottom Left) New bone formation within the cement. Bottom Right) New bone formation on the HydroSet™ surface.

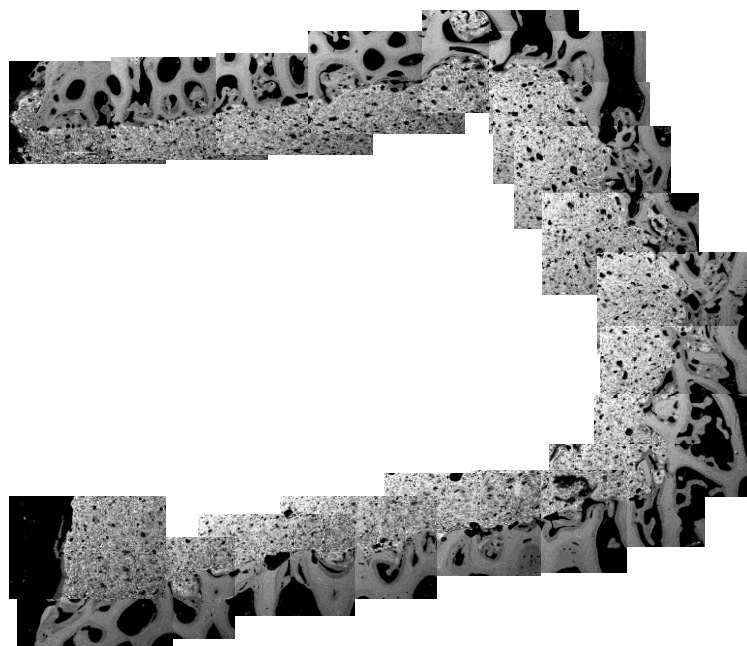


FIGURE 7: A mosaic SEM image showing bone in contact with the cement surface around the length of the defect.

Figure 7 shows a mosaic of SEM images overlaid to produce a continuous image of the cement/bone interface. The images show disordered bone formation surrounding both the cement and the original trabecular struts adjacent to the implant site.

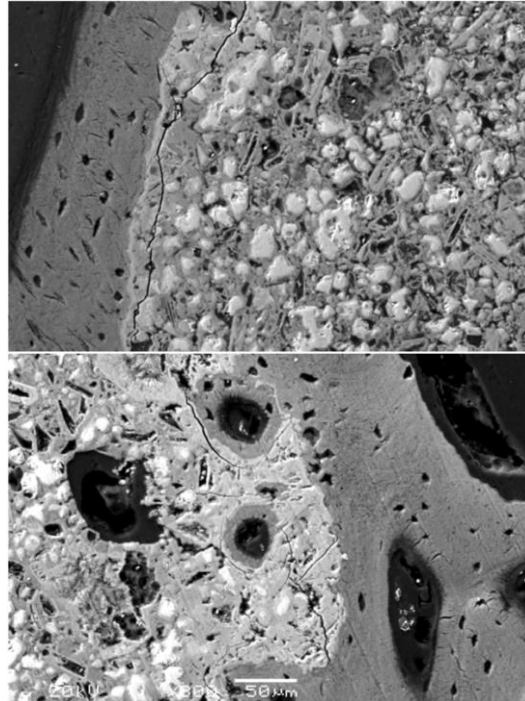


FIGURE 8: SEM micrographs showing bone in direct contact with the cement surface with good integration. Cracks apparent on the SEM images are artefacts and appeared during preparation.

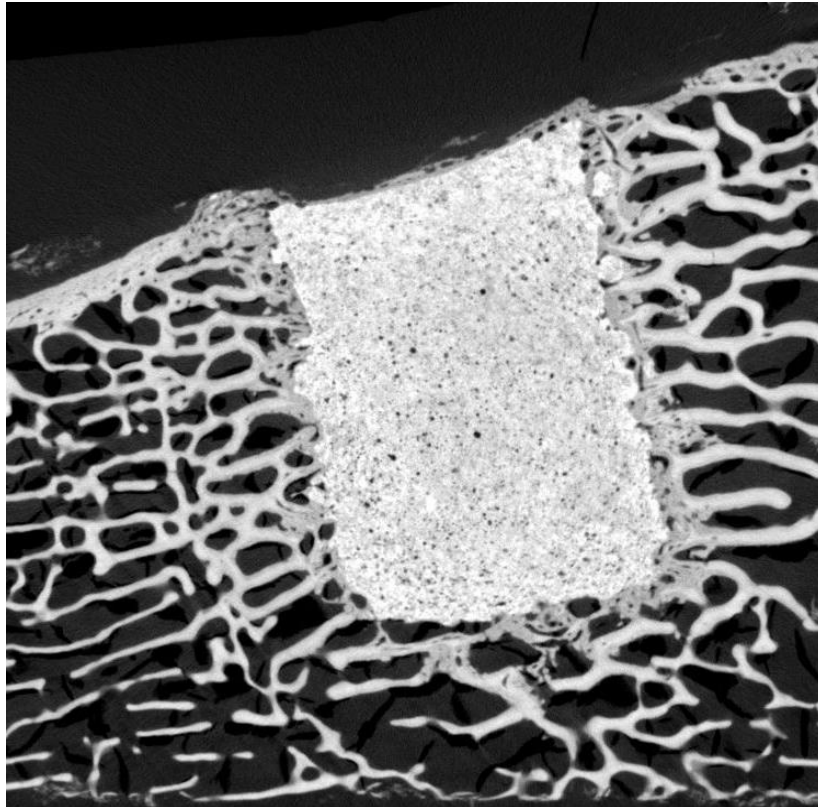


FIGURE 9: X-ray micrograph of internal face of implanted HydroSet™ within bone.

Discussion

Hydroxyapatite was thought to be the only phase that forms during the setting of HydroSet™ the cement phase²⁶. However, the ³¹P MAS NMR and XRD results shown here revealed that the cement additionally forms octacalcium phosphate. Strong evidence of OCP on the ³¹P MAS NMR spectra is a peak at 3.6 ppm which would be expected if OCP was present. The other chemical shift for OCP at 3.3, 2.0 and -0.2 ppm are partially obscured overlapping with other signals in the narrow range between -1.4 and 5.0 ppm. The ³¹P MAS NMR results are in agreement with the XRD data on OCP as described in the previous section.

Tseng, et al.²² showed the transition from octacalcium phosphate to hydroxyapatite via ³¹P MAS NMR over 96 hours immersed in solution. The authors showed the initial pure octacalcium phosphate spectrum with resonances at 3.7, 3.3, 2.0 and -0.2 ppm and then demonstrated how the resonances at -0.2, 2.0 and 3.7 ppm gradually decreased over six hours finally reaching relatively low intensity. Eventually only one resonance was remained present at around 3.0 ppm, of hydroxyapatite. The rate of this transition is likely to be affected by the temperature, pH and ion constituents of the immersion solution. It is likely that the samples containing octacalcium phosphate in our results are already in the process of transition into apatite since the spectra shown more closely match the five or six hour samples of Tseng *et al*, explaining the lack of relative intensity of the resonances at -0.2, 2.0 and 3.7 ppm.

It is known that OCP often acts as a precursor to hydroxyapatite because OCP nucleates and grows more readily than hydroxyapatite and its formation is kinetically more favourable than that of hydroxyapatite under certain conditions²⁷. Previous studies have shown this occurring *in vitro*^{27, 28} and there have been studies finding OCP during osteogenesis^{4, 29}. However, it is not known from these results if the OCP forming is a precursor to the hydroxyapatite formation in this particular cement, or if it forms in addition to the hydroxyapatite.

OCP forming calcium phosphate cements have been reported previously³⁰ but this phase was not considered as a precursor species to hydroxyapatite in cements; although this may have just been due to difficulty in identification of the OCP phase. The main reason why no studies have previously found octacalcium phosphate in this setting reaction is probably because of the typically investigators study a reasonably narrow two theta range in copper X-ray diffraction analysis. Octacalcium phosphate and hydroxyapatite, due to their similar crystal structures, have similar diffraction patterns. The main difference is a line at 4.7 degrees two theta present in only the OCP pattern.

The X-ray diffraction and ³¹P MAS NMR results show the octacalcium phosphate transforming to hydroxyapatite with 28 days in Tris buffer solution. Whilst the DCPD fully dissolves between 1 day and 7 days, the tetracalcium phosphate is still present at 28 days.

It is believed that the reaction from OCP to hydroxyapatite can occur through crystal transition as well as through dissolution and reprecipitation. This crystal transition route is supported by a number of studies showing intercrystalline mixtures of OCP and hydroxyapatite^{4,31}. A crystal of partly hydrolysed OCP when studied on XRD gave the pattern of OCP and hydroxyapatite with the *b*- and *c*- axis collinear⁴. This suggests an intercrystalline mixture of the two crystals given the similarity in their crystal structure. The proposed setting reaction of HydroSet™ is listed below:

1. $2\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} + 2\text{Ca}_4(\text{PO}_4)_2\text{O} + \text{H}_2\text{O} \rightarrow \text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O} + 2\text{Ca}^{2+} + 2(\text{OH})^-$
2. $\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O} + 2\text{Ca}^{2+} + 2(\text{OH})^- \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 2\text{H}^+ + 5\text{H}_2\text{O}$

The ³¹P MAS NMR was also very useful for phase identification and the detection of impurities. Whilst the formulation of HydroSet™ is only supposed to contain DCPD and TTCP, the NMR showed the presence of a small fraction of DCPA that was not detected on the X-ray diffraction. Another interesting result is the amount of time the tetracalcium phosphate takes to fully react to form the cement phase. The two starting reagents are added together in an overall calcium to phosphorus ratio of 1.67 to favour apatite formation²⁶. This could be due to the relative particle sizes of the two starting reagents or lack of water penetrating the cement cylinder causing the dissolution of the tetracalcium phosphate and precipitation of the cement phase, as it has been shown particle size can have a large influence on the reactivity of starting components in calcium phosphate cements³². Hannink *et al.* showed that the TTCP in the cement remains until twenty six weeks after implantation *in vivo*¹⁹. The scanning electron micrographs (Figure 8) of the implant surface show residual granules within the cement block in the implant site, it is possible that these are remnant unreacted TTCP particles.

Stryker, the manufacturers of HydroSet™ state that HydroSet™ has an initial setting time of 4.5 minutes and a final setting time of 8.5 minutes³³ whereas the results using the Gilmore needle test give an initial setting time of 6 minutes and final setting time of 19 minutes. This difference is because the manufacturers use a different method to measure the initial and final setting times. This highlights that setting time is a difficult thing to assess and measure and especially apply this to a clinically meaningful context. The assessment should measure whether the material sets well enough *in vivo* to allow the clinician to easily implant it for a specific application, whilst not causing any negative effects such as washout or disfigurement.

The compressive strength of the cement showed a maximum compressive strength of 25 MPa at one and twenty-eight days. Interestingly a decrease in compressive strength between these two points at seven days to 13 MPa, which correlates with and perhaps is caused by the hydrolysis of OCP to hydroxyapatite, identified on the phase analysis. A study performed by Clarkin *et al.* studied the mechanical properties of HydroSet™ and found compressive strengths of between 18 and 24 MPa,

comparable to this study²⁶. A second study performed by Van Lieshout, *et al.*³⁴ showed a similar compressive strength of around 11 MPa after three days of storage in water, a similar result to that found here. With respect to the relevance of this to clinical use these levels are approximately similar to vertebral bone²⁶ and trabecular bone³⁵.

The *in vivo* results show very high rates of osseointegration for HydroSet™. The measurement of graft-to-bone contact measured from histological images give a mean percentage contact of 92.5 %. This is supported in the microscopy, SEM and X-ray microtomography results. The photomicrographs of histological sections show the interface between the cement and original trabecula bone struts. New bone formation is clearly distinguishable from these images, this is especially highlighted in Figure 6 where new bone is clearly labelled forming on both the cement surface and trabeculae present before surgery. Similarly the scanning electron micrographs show a strong union between the bone and cement surface. High osseointegration is important for stability of the bone graft within the implant site, which is especially important for these types of materials as they are frequently used to augment metal screws or augment an arthroplasty procedure. With respect to other calcium phosphate based grafting materials, a study by Chan *et al.* using the same animal model showed bone-to-graft contact of <50% for a hydroxyapatite based granular bone substitute implanted for a similar period³⁶.

A study performed by Hannink *et al.* implanted HydroSet™ into the distal femur of rabbits¹⁹. The authors measured percentage bone contact between the cement and new bone at six, twenty six and fifty two weeks of implantation. Percentage contact was approximately 80-85% bone-to-graft contact, for all time points measured. The authors also compared Norian SRS and BoneSource in the same study and found similar levels of bone contact for these too. These levels are similar to what was measured here and therefore suggest high levels of osseointegration are associated with calcium phosphate cements. It should be noted that all of the cements studied consist of mixtures of TTCP and DCPD¹⁹. A study by Flautre *et al.*³⁷ implanted four compositions of injectable calcium phosphate cement in an distal femur ovine animal model. The authors compared a Brushite forming calcium phosphate cement (BCPC), an empty site and defects filled with autologous bone. Although the authors did not directly measure bone-to-graft contact, the microradiographs presented in the paper showed very limited osseointegration in the implant site. The authors found bone apposition rates of 1.5 $\mu\text{m day}^{-1}$ for the BCPC, 1.2 $\mu\text{m day}^{-1}$ for the empty site and 1.1 $\mu\text{m day}^{-1}$ for the autograft at 24 weeks. The ovine model is believed to accurately represent bone apposition rates compared to that in humans²³. The bone apposition rate, in the results presented here, was measured as 1.191 ($\pm$ 0.345) $\mu\text{m day}^{-1}$, with respect to other bone substitutes this value is comparable to other injectable calcium phosphate cements implanted within sheep.

The considerably higher *in vivo* compressive strength compared to that measured *in vitro* was surprising. The highest *in vitro* compressive strength measurement was 25 MPa whereas *in vivo* at twelve weeks this value was 94 MPa. The cause of this increase is likely due to the ingrowth of bone (shown on the histology) into the cement pores that would have been ‘unfilled’ *in vitro*. This explanation is supported by the results of Van Lieshout, *et al.*³⁴ who showed that porosity and compressive strength are negatively correlated. This result also raises questions with respect to the *in vitro* measurements of bone cements and their validity. Calcium phosphate cements are sometimes dismissed for certain applications due to their relatively low, *in vitro*, mechanical properties in favour of stronger acrylic or ionomer bone cements. These results suggest that *in vivo* these cements are actually much stronger, due to their ability to osseointegrate, than previously thought.

One of the most significant aspects of this paper is the discovery of the formation of octacalcium phosphate. The HydroSet™ starting reagents are identical to those originally formulated in CPCs (and

is commonly used in commercial examples) it is likely that many other CPC formulations also form OCP but it has not been previously identified. Octacalcium phosphate has been shown to enhance bone formation *in vivo*^{38, 39}, have a positive impact on cell proliferation⁴⁰, and resorbs more readily than hydroxyapatite⁴¹.

Conclusions

The paper presented phase evolution, properties and *in vivo* study of the HydroSet™ cement. In addition to apatite phase known to form as a result of the setting, the phase of the OCP was also found at early stage in the cement. This implies that a number of other cements including commercially available that use similar starting calcium phosphates form OCP during the setting. In HydroSet™ *in vitro* results showed an initial setting time of 6 minutes, a final setting time of 19 minutes and maximum compressive strength of 25MPa. *In vivo* results showed bone apposition rates of 1.191 ($\pm$ 0.345) $\mu\text{m day}^{-1}$, bone-to-graft contact of 92.5 % and an *in vivo* compressive strength of 95 MPa. Comparisons on the *in vitro* and *in vivo* showed that HydroSet™ is considerably stronger *in vivo* compared to *in vitro*, the cause of this attributed to bone formation within the cement pores and a high degree of osseointegration.

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