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The effect of amorphous pyrophosphate on calcium phosphate cement resorption and bone generation☆

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ABSTRACT

Pyrophosphate ions are both inhibitors of HA formation and substrates for phosphatase enzymes. Unlike polyphosphates their hydrolysis results simultaneously in the complete loss of mineral formation inhibition and a localised elevation in orthophosphate ion concentration. Despite recent advances in our knowledge of the role of the pyrophosphate ion, very little is known about the effects of pyrophosphate on bone formation and even less is known about its local delivery. In this work we first developed a self setting pyrophosphate based calcium cement system with appropriate handling properties and then compared its in vivo degradation properties with those of a non-pyrophosphate containing control. Contrary to expectation, the presence of the pyrophosphate phase in the cement matrix did not inhibit mineralisation of the healing bone around the implant, but actually appeared to stimulate it. *In vitro* evidence suggested that enzymatic action accelerated dissolution of the inorganic pyrophosphate ions, causing a simultaneous loss of their mineralisation inhibition and a localised rise in supersaturation with respect to HA. This is thought to be a rare example of a biologically responsive inorganic material and these materials seem to be worthy of further investigation. Bioceramics to date have mainly been limited to orthophosphate, silicate and carbonate salts of calcium, here we report the successful application of a pyrophosphate material as a degradable osteoconductive bone repair cement.

1. Introduction

Calcium orthophosphate based bone graft replacements have been investigated as potential bone graft replacements since 1920 when tricalcium phosphate was injected into an animal model [1]. During the 70's there was a concerted effort to develop new calcium phosphate grafts based on tricalcium phosphate or hydroxyapatite (HA) monoliths [2,3]. Despite 40 years of further research, HA has not replaced autologous tissue as the surgical 'gold-standard'. The clinical success of HA can be attributed to the fact that it forms a bond with both hard and soft tissues and is osteoconductive [4]. One of the drawbacks of using HA is that, in its dense form, it remains in the body for a prolonged period following implantation [5] and its brittle nature means that it presents a long-standing risk of catastrophic failure [6]. Some workers have attempted to solve this problem by incorporating macroporosity (>200 μm) to allow bone in-growth [7]. While the original rationale behind using HA as bone replacements was clear, the principle itself is somewhat crude. The HA crystallites in bone are typically nanoscopic (100 nm in length, thickness 4–6 nm, and breadth 30–45 nm [8]) and incorporate numerous substitutions, whereas sintered HA and β-TCP (β-tricalcium phosphate; $Ca_3(PO_4)_2$) are microcrystalline and consequently exhibit a correspondingly low specific surface area. Furthermore, although bone is formed of 60–70 vol% HA, the collagen matrix and other proteins in bone play important biological and structural roles. Therefore, other than a compositional similarity, HA bone graft substitute typically has little mechanical or biological similarity to bone. Consequently, a number of other groups have attempted to

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develop new grafting materials using other osteoconductive calcium salts more soluble in physiological conditions such as brushite [9–12]. Although some success has been reported, brushite grafts tend to hydrolyse to form HA [13], which can also result in long-term implant stability and therefore present a prolonged risk of implant failure. There are several reports in the literature of brushite-based calcium phosphate cements being chemically modified to prevent the hydrolysis reaction from occurring. Newberryite [14], for example, has been added to brushite cement and has been shown to prevent hydrolysis from occurring *in vitro*. Unfortunately, these results could not be reproduced *in vivo* [15]. We have also modified brushite-based cements using pyrophosphoric acid as a reactant [12,16,17]. This modification enabled the production of a cement containing an amorphous pyrophosphate phase, which was shown to prevent the hydrolysis reaction for over 90 days *in vitro* [16]. Pyro or diphosphate ($P_2O_7^{4-}$) ions are inhibitors of HA crystal formation and are essential in the control of biomineralisation [17–19], their potent mineralisation inhibiting activity has been exploited to soften water. Importantly, although pyrophosphates inhibit mineral formation [18], active osteoblasts secrete alkaline phosphatase (ALP), which can hydrolyse pyrophosphate ions (Equation (1)), which simultaneously causes loss of this ion's potent inhibiting effect on HA formation and supersaturates the extra-cellular fluid with orthophosphates that induce mineralisation [19]. This mechanism has been shown to be critical to the control of bone mineralisation. With the exception of the highly insoluble and thermally stable phase β-dicalcium pyrophosphate [20], the pyrophosphates are relatively unexplored in bone graft replacement applications, despite decades of research demonstrating the role of this ion in bone.

$$P_2O_7^{4-} + H_2O \rightarrow 2PO_4^{3-} + 2H^+ \quad (1)$$

Here we have investigated whether the amorphous pyrophosphate phase can prevent brushite hydrolysis and consequently long-term cement stability *in vivo*. Following periods of implantation, cement samples were retrieved and characterised with respect to composition using ion chromatography and X-ray diffraction. Sections were made of the implant site and the extent of implant resorption was determined using histomorphometry. Finally, an *in vitro* model was used to systematically evaluate how the enzyme alkaline phosphatase interacts with amorphous calcium pyrophosphate phase.

2. Materials and methods

2.1. Cement formulation

A systematic study was undertaken to determine the liquid compositions at which setting pyrophosphate cements could be produced while maximising amorphous calcium pyrophosphate content. All cement pastes were formed from the mixture of β-TCP (Plasma-Biotal, Derbyshire, UK), pyrophosphoric acid, orthophosphoric acid (Sigma–Aldrich, Gillingham, UK) and double distilled water at a constant powder to liquid ratio (P:L) of 1.50 g/mL. Cement pastes were formed where the water constituted of between 0 and 90 wt% of the liquid phase (at 5 wt% increments). At each water content, the proportions of pyrophosphoric and orthophosphoric acids were varied so that every possible liquid composition (at 5 wt% increments) was produced. To find the final setting times exhibited by the cements a needle (1.05 mm diameter and 454 g mass) was applied to the surface of the cement. The point at which this needle no longer indented the surface was the final setting time. All measurements were made in ambient conditions and were repeated three times for each cement formulation investigated. From the resultant data the compositions were grouped into those that when mixed with β-TCP (powder to liquid ratio 1.50 g/mL) set in a.) less than 30 s, b.) 30 s to 30 min, c.) 30 min to less than 24 h and d.) non-setting formulations. A ternary plot was then constructed to show the liquid compositions resulting in the formation of cements setting fully in these ranges. Once the ternary plot was completed the entire experiment was repeated to ensure accuracy.

2.2. X-ray diffraction and nuclear magnetic resonance

The compositions of the hardened cements were determined using X-ray diffraction (XRD) and nuclear magnetic resonance (NMR). Dried cement samples were powdered using a pestle and mortar. For XRD, the powder was placed between two pieces of magic™ tape (3M, Minnesota, USA) and attached to the sample holder, which was mounted in the X-ray diffractometer (Siemens D5000, Munich, Germany). X-ray powder diffraction data were collected using Cu $K\alpha_1$ radiation from a powder X-ray diffractometer, fitted with a Ge primary beam monochromator and aligned in transmission mode. For phase analysis each data set was collected from 5° to 100° 2θ with step size 0.02°, each step requiring 9 s, such that patterns required approximately 12 h to collect. A multiphase, whole pattern Rietveld analysis using the General Structure Analysis Suite (GSAS) of programs was employed to determine the crystalline phase compositions of the samples. Structural models for $CaHPO_4 \cdot 2H_2O$, $CaHPO_4$, β-$Ca_3(PO_4)_2$, β-$Ca_2(P_2O_7)$, $Ca_2P_2O_7 \cdot 2H_2O$, and $Ca_{10}(PO_4)_6OH_2$ [21–26] were obtained from the literature references and a linear interpolated background function was utilised, together with a pseudovoigt peak shape function. The pyrophosphate contents of cement formulations one to eleven were determined by quantitative NMR of powdered cement. High-resolution solid state ^{31}P NMR experiments were performed at 121.51 MHz using an NMR spectrometer (CMX Infinity 300 spectrometer, Chemagnetics, CO, USA) with a Chemagnetics 4 mm triple-resonance magic angle spinning (MAS) probe. All spectra were recorded using direct polarisation with ^1H decoupling (with a decoupling field strength of 100 kHz), magic angle spinning (with a spinning frequency of 7000 ± 2 Hz) and a set sample temperature of 25 °C. The spectra were referenced relative to 85 wt% orthophosphoric acid at 0 ppm. To ensure that the ^{31}P MAS NMR spectra of the cement samples were quantitative ^{31}P spin-lattice relaxation times (T_1) were first determined for samples of the components of the cements using the saturation-recovery technique. The high-resolution solid state ^{31}P NMR spectra of the cement samples were then recorded using a recycle delay of greater than five times the longest value T_1 determined for the individual cement components. The composition of the cements could then be determined by fitting the spectra using the spectrometer software to obtain reliable peak intensities.

2.3. Determination of orthophosphate:pyrophosphate

In order to determine the proportions of the hardened cement consisting of orthophosphate and pyrophosphate phases, approximately 25 mg of cement were dissolved in 100 mL nitric acid (100 mM). The resulting solution was diluted ten times with double distilled water before analysis using an ion chromatography system (ICS-2500, Dionex, Sunnyvale, CA, USA) equipped with an appropriate analytical column (IonPac AS16, Dionex, Sunnyvale, CA, USA).

2.4. In vivo experimentation

To evaluate biological response to the material and cement resorption, samples were implanted in an ovine model. The pyrophosphate modified cement used in the study was formed by combining β-TCP with pyrophosphoric acid (540 mg) and double distilled water (720 mg) at a powder to liquid ratio of 2.25 g/mL. The orthophosphate brushite cement was formed by the combination of β-TCP with orthophosphoric acid (2 M) containing trisodium citrate (50 mM) at a powder to liquid ratio of 1.75 g/mL. The cements were cast into PTFE split moulds to form hardened cement cylinders (diameter 6.4 mm and length 12 mm). The samples were stored at 37 °C and 100% relative humidity for 24 h prior to sterilisation by gamma irradiation. The cement cylinders (four replicates per time-point) were implanted into the tibiae of eighteen female Blue Faced Leicester cross Suffolk sheep of approximately four years in age according to a randomisation schedule. Briefly, anaesthesia was induced with thiopentone sodium (5%) which was administered 'to effect' by intravenous injection. An incision was made in the medial side of the proximal tibia. A defect (6.4 mm diameter, 12 mm deep) was then created with an orthopaedic drill, with at least one intermediate-sized drill used to reduce the chance of thermal necrosis to the bone. Bone debris was removed by saline irrigation and the defect flushed with local anaesthetic. The defects were filled with test material according to the randomisation schedule. To enable the location of the defect site two K-wires were inserted either side of it. Each K-wire was implanted at a distance of approximately 8 mm from the centre of the implant so that the wires and the centre of the implant were aligned in a straight-line. The K-wires were inserted at a depth not greater than 30 mm. This procedure was then repeated in the bilateral leg. The muscle, fascia, subcutaneous tissue and skin were closed in a standard surgical manner using resorbable sutures. Calcein was administered 24–48 h (10 mg/kg) after surgery. Alizarin C-one marker was administered at one, two and four months following implantation in the sheep (30 mg/kg) for the three, six and twelve month time-points, respectively. Oxytetracycline was given (30 mg/kg) one week prior to sacrifice. All animals were euthanised using Pentoject. Following gross pathology observations the implant site/tibial plateau was removed and the surrounding bone trimmed down. Some surrounding undamaged bone was retained so that histological analysis of the undamaged bone could be performed. All samples were fixed in alcohol (70%) at 4 °C. The implants were then bisected into two halves using a low-speed diamond saw. The cortical half of the explant was embedded into a methyl methacrylate resin. The resin blocks were sectioned using the Leica diamond microtome saw. Sections were cut (200 μm thickness) and adhered to plastic slides. The sections were ground down to approximately 100 μm. Two resin sections were prepared, one for evaluation by fluorescent microscopy, the other was stained with