Cements from nanocrystalline hydroxyapatite

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Calcium phosphate cements are used as bone substitute materials because they may be moulded to fill a void or defect in bone and are osteoconductive. Although apatite cements are stronger than brushite cements, they are potentially less resorbable in vivo. Brushite cements are three-component systems whereby phosphate ions and water react with a soluble calcium phosphate to form brushite (CaHPO₄·2H₂O). Previously reported brushite cement formulations set following the mixture of a calcium phosphate, such as β-tricalcium phosphate (β-TCP), with an acidic component such as H₃PO₄ or monocalcium phosphate monohydrate (MCPM). Due to its low solubility, hydroxyapatite (HA) is yet to be reported as a reactive component in calcium phosphate cement systems. Here we report a new cement system setting to form a matrix consisting predominantly of brushite following the mixture of phosphoric acid with nanocrystalline HA. As a result of the relative ease with which ionic substitutions may be made in apatite this route may offer a novel way to control cement composition or setting characteristics. Since kinetic solubility is dependent on particle size and precipitation temperature is known to affect precipitated HA crystal size, the phase composition and mechanical properties of cements made from HA precipitated at temperatures between 4 and 60 °C were investigated.

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1. Introduction
There has been great interest in calcium phosphate cements as bone substitute materials [1–4] because they can be moulded to fill a void or defect and are osteoconductive [5–7]. In 1985, Brown and Chow reported [8–10] a calcium phosphate paste that set and hardened to form a cement at ambient temperature. This cement was composed of mostly apatite, which can have a low rate of resorption in vivo [4, 11, 12]. Shortly after 1987 Lemaître et al. [13] reported another calcium phosphate cement system whereby cement consisted of predominantly brushite. Brushite cements are thought to be more soluble than apatite cements in physiological conditions and hence resorbable in vivo [4, 11, 14].

Brushite cements are three-component systems whereby phosphate ions and water react with a calcium phosphate to form brushite (CaHPO₄·2H₂O). In previous reports the calcium phosphate component has been β-tricalcium phosphate (β-TCP) reacted with monocalcium phosphate monohydrate (MCPM), Equation 1, or simply phosphoric acid solution, Equation 2 [15, 16].

\[
\beta-\text{Ca}_3(\text{PO}_4)_2 + \text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} + 7\text{H}_2\text{O} \rightarrow 4\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \quad (1)
\]

\[
\beta-\text{Ca}_3(\text{PO}_4)_2 + \text{H}_2\text{PO}_4 + 6\text{H}_2\text{O} \rightarrow 3\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \quad (2)
\]

Hydroxyapatite (HA) has not previously been reported as a brushite cement reactant. HA is the least soluble calcium orthophosphate at physiological conditions. However, at pH 4.2 in the system Ca(OH)₂–H₃PO₄–H₂O at 25 °C it is as soluble as brushite [10, 14, 17]. Depending on reaction conditions, it might be expected that cements formed from HA could be biphasic: consisting of unreacted HA in a brushite matrix. Factors that would affect extent of reaction are relative concentration of reactants, temperature and the particle size of solid reactants [18]. Greater kinetic solubility may be achieved by increasing HA surface area. Surface area may be increased by reducing the particle size of solid reactants by milling/grinding or altering precipitation conditions. Precipitated HA has a

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greater specific surface area than HA produced by thermal treatment. Furthermore, precipitation temperature is well known to affect the degree of supersaturation and hence crystal size [19].

We report the effect of HA precipitation temperature on composition and mechanical properties of cements formed with 3M phosphoric acid solution. Mixing precipitated HA and phosphoric acid produced cements predominantly composed of brushite.

2. Methods and materials
Nanocrystalline HA was precipitated following Jarcho et al. [20]. Briefly, 1.4 mol of calcium nitrate (Ca(NO3)2) (Sigma) were dissolved 900 ml of double distilled water (DDW). The pH of this solution was brought between 11 and 12 by adding concentrated aqueous ammonia (NH3·H2O) (Fisher) and the solution diluted to 1.81. Diammonium phosphate solution was prepared by adding 1 mol (Sigma) to 1.51 of DDW. Concentrated aqueous ammonia was added to this solution to bring pH between 11 and 12. The solution was diluted to 3.21. The calcium nitrate solution was vigorously stirred and temperature was maintained at 4, 20 or 60°C and the ammonium phosphate solution added drop wise over 30–40 min. The reaction mixture was stirred overnight (> 12 h) and then centrifuged (2000 rpm for 10 min) and the supernant decanted and the precipitate was re-suspended in DDW. This washing procedure was repeated twice more. Centrifuged precipitate was dried at 75°C, ground with a pestle and mortar and sieved to a size of < 300 μm in diameter. The calcium phosphate ratio (Ca/P) of precipitated HA was calculated using chemical titration and photometry. Dried HA precipitate (18 mg) was dissolved in 1 ml of 1 M hydrochloric acid solution (Sigma). The amount of calcium present was determined by titrating the dissolved HA solution against a 5 mM solution of ethylenediaminetetraacetic acid. The phosphate in dissolved HA solution was complexed with molybdate–vanadate reagent and its quantity calculated photometrically at 400 nm.

Cements were formed with precipitated HA and 3 M phosphoric acid (Sigma) containing 50 mM sodium citrate (Sigma) solution. Cements were mixed at a powder to liquid ratio of 1.5 g/ml. X-ray diffraction patterns of HA precipitates and set cements were recorded from 20 = 20–40° with a step size of 0.02° and a count time of 5–10 s/step (D5005 Siemens, Karlsruhe, Germany). The phase composition was determined by means of JCPDS reference patterns for β-TCP, HA and brushite. Crystal sizes and quantitative phase compositions of materials were calculated by means of total Rietveld refinement analyses with TOPAS software (Bruker AXS). As references, the system internal database structures of β-TCP, HA and brushite were used together with a Chebyshev fourth order background model and a Cu Kα emission profile.

Scanning electron microscopy (SEM) was carried out with a JEOL JSM 6300 at an accelerating voltage of 20 kV. Transmission electron microscopy (TEM) was performed with a JEOL JEM 4000FX. Precipitates were suspended in 100% ethanol. Once precipitates had been suspended, 500 μl were transferred to a carbon-coated grid and suspended precipitates stored in ambient conditions to allow ethanol to evaporate leaving an even distribution of dried precipitate over the carbon-coated grid surface. TEM was performed at an accelerating voltage of 80 kV. Initial and final setting times were measured using the Gilmore needle technique [21] at ambient conditions. The time elapsing between cement mixing and no visible impression being made on the cement surface by the needles was recorded. Density of cement products was measured using helium pycnometry (Accupyc 1330, Micromeritics). Relative density was calculated by dividing the apparent density, as determined from geometry and mass of cements, by measured density.

Cylindrical cement specimens were formed with a diameter of 6 mm and a height of 12 mm. These cements were stored under ambient conditions for 24 h prior to testing. Cements were loaded under compression until failure (Instron 5544) at a crosshead speed of 1 mm/min, with a 2 kN load cell, to determine compressive strength.

3. Results
Total Rietveld refinement analysis of XRD data revealed that the precipitated calcium phosphate was low crystallinity phase pure HA with no other calcium phosphate phases detectable (Table 1). The crystals of this HA varied in size as precipitation temperature was altered. HA precipitated at lower temperature had smaller crystals; HA precipitated at 4°C had a mean crystal size 34.8 (± 6.1) nm whilst HA precipitated at 60°C had a mean size around double the size, 60.8 (± 5.9) nm. As determined from XRD data cement formed with precipitated HA was made up of a combination of predominantly brushite and unreacted HA. HA remaining in the cement had crystal sizes of approximately half the size observed in the HA precipitate, for example, HA precipitated at 20°C had crystals 44.2 (± 3.9) nm whilst unreacted HA in the cement was 16.6 (± 1.5) nm.

<table>
<thead>
<tr>
<th>HA precipitation temperature (°C)</th>
<th>Calcium phosphate precipitate</th>
<th>Cement</th>
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<tbody>
<tr>
<td>wt % HA</td>
<td>HA crystal size (nm)</td>
<td>wt % HA</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>34.8 (± 6.1)</td>
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<tr>
<td>20</td>
<td>100</td>
<td>44.2 (± 3.9)</td>
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<tr>
<td>60</td>
<td>100</td>
<td>60.8 (± 5.9)</td>
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