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In vitro degradation and *in vivo* resorption of dicalcium phosphate cement based grafts

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ABSTRACT

There are two types of DCP: dihydrated (brushite) and anhydrous (monetite). After implantation, brushite converts to hydroxyapatite (HA) which resorbs very slowly. This conversion is not observed after implantation of monetite cements and result in a greater of resorption. The precise mechanisms of resorption and degradation however of these ceramics remain uncertain. This study was designed to investigate the effect of: porosity, surface area and hydration on *in vitro* degradation and *in vivo* resorption of DCP. Brushite and two types of monetite cement based grafts (produced by wet and dry thermal conversion) were aged in phosphate buffered saline (PBS) and bovine serum solutions *in vitro* and were implanted subcutaneously in rats. Here we show that for high relative porosity grafts (50–65%), solubility and surface area does not play a significant role towards *in vitro* mass loss with disintegration and fragmentation being the main factors dictating mass loss. For grafts having lower relative porosity (35–45%), solubility plays a more crucial role in mass loss during *in vitro* ageing and *in vivo* resorption. Also, serum inhibited dissolution and the formation of HA in brushite cements. However, when aged in PBS, brushite undergoes phase conversion to a mixture of octacalcium phosphate (OCP) and HA. This phase conversion was not observed for monetite upon ageing (in both serum and PBS) or in subcutaneous implantation. This study provides greater understanding of the degradation and resorption process of DCP based grafts, allowing us to prepare bone replacement materials with more predictable resorption profiles.

1. Introduction

Dicalcium phosphates (DCP) are calcium phosphates of great interest for orthopaedic and dental applications. Brushite cements set via a dissolution/precipitation process at low pH (<6) [1,2]. One of methods by which brushite cements can be prepared is by mixing an acidic calcium phosphate such as monocalcium phosphate monohydrate (MCPM) and a basic calcium phosphate like beta tricalcium phosphate (β-TCP) with water. This method results in a mouldable paste that sets into a solid cementitious material composed mainly of dicalcium phosphate dihydrate (DCPD) [3–5]. Brushite cements can also be utilised as precursors to the anhydrous form of dicalcium phosphate (DCPA), also known as monetite. Brushite crystals when heated above 60 °C start to dehydrate into monetite [6], and if moisture is maintained during the heating process (as in autoclaving) then bulk shrinkage is prevented and an increase in the internal pore size is observed [7]. This conversion into monetite can also be carried out by dry heating the preset brushite cements [8]. These wet and dry heat conversions of brushite to monetite results in two materials that are chemically very similar yet differ with respect to physical properties (total porosity, pore size distribution, density and surface area) [8,9].

The success of bone replacement procedures is limited by the low or negligible resorption rates associated with the use of calcium phosphate cements [2]. A significant reduction in the rate of resorption is frequently reported due to the phase conversion of brushite to hydroxyapatite (HA) [10,11]. It has been observed that monetite does not re-precipitate into HA *in vivo*, and recent research demonstrates its good osteoconductive and osteoinductive properties [8,9,12–15].

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Studies investigating behaviour of brushite cements after implantation or immersion in aqueous media have reported resorption, disintegration or long-term stability [16–18]. Brushite cements have been shown to exhibit a decrease in mass, an increase in porosity, and a deterioration in mechanical properties upon *in vitro* incubation [19]. It has been reported that cement porosity, as well as the properties of surrounding medium and the rate of fluid exchange affects initial brushite resorption [20]. Disintegration and fragmentation of cement matrix rather than simple dissolution can also contribute to mass loss during brushite incubation *in vitro* [20].

Brushite cements have been shown to experience an initially linear degradation rate of 0.25 mm per week once implanted *in vivo* [16]. However, degradation rate is dependent on a variety of factors such as: cement physico-chemical properties, species of animal in which implanted site of implantation and blood flow. After implantation, during initial few weeks brushite cements appear to resorb by disintegration, simple dissolution and cellular activity (macrophages and osteoclasts) [17,21,22]. It has also been observed that serum can adsorb onto cement surface altering the interfacial properties promoting brushite resorption *in vivo* and *in vitro* [20]. The resorption mechanism of monetite is similar to that observed for brushite cement grafts, in that it is mainly mediated by cellular activity and simple dissolution [23]. Recent studies have shown that monetite grafts produced by autoclaving of preset brushite cements appear to resorb more in comparison to the original brushite grafts [9,24].

In this study, brushite cement grafts were prepared and monetite grafts obtained from them via wet heat and dry heat conversion. The *in vitro* and *in vivo* behaviour of these grafts was investigated by ageing in phosphate buffered saline (PBS) and bovine serum solutions and also subcutaneous implantations in rats. We attempted to discern the effect of DCP hydration, porosity and surface area on *in vitro* and *in vivo* degradation and resorption.

2. Method and materials

2.1. Synthesis

Brushite cement grafts were prepared with a mixture of β-TCP (Merck) and commercially available monocalcium calcium phosphate monohydrate (MCPM) (ABCR, GmbH & Co.KG) using a ratio of 1.2 to 1 respectively. In order to investigate the effect of powder to liquid (P/L) ratio on physical properties and degradation, brushite and monetite cements were produced at P/L mixing ratio of 3 and 1 g/ml. The powders were hand ground with a pestle and mortar and cement pastes prepared by mixing the powder with appropriate amount of distilled water on a glass slab for 20 s. Once all of the powder was combined with the liquid, the cement paste was kneaded for a further 30 s. The manipulated cement slurry was cast into a polytetrafluoroethylene (PTFE) split mould forming hardened cement cylinders ∅ (∼12 mm (height) × 6 mm (diameter)). The cylinders were allowed to set for 24 h at 37 °C ± 1 °C in a vacuum desiccator to form hard brushite. At the end of the incubation period, the samples were removed from the mould and weighed until constant mass was reached. Five different batches of thirty cylinders each were produced to obtain a total of one hundred and fifty cylinders with 3 and 1 P/L ratios. Even though samples were prepared using same protocol, the sample assignment was randomized to minimise variations.

Monetite cement grafts ($n = 70$ in total) were synthesized by conversion of the preset brushite cement cylinders utilising two different methods: thermal and hydrothermal conversion. For thermal conversion, the brushite cylinders ($n = 35$) were dry heated at 250 °C for 30 min under vacuum (80 mTorr). Hydrothermal transformation was performed with the brushite cylinders ($n = 35$) being autoclaved at sterilizing conditions (120 °C, 100% humidity and 15 psi, for 30 min).

2.2. Characterisation

The phase purity of the prepared brushite and the monetite grafts was confirmed using X-ray diffraction (XRD). XRD data was collected (Bruker Discover D8 diffractometer) from the surface of the graft materials with Ni filtered CuKα radiation ($\lambda = 1.54$ Å) with a two dimensional VANTEC area detector at 40 kV and 40 mA. A step size of 0.02° was used to measure from 10° to 50° 2θ over 3 frames with a count time of 300 s per frame. The phase composition was compared and confirmed with the International Centre for Diffraction Data reference patterns for brushite (PDF Ref. 09-0077) and monetite (PDF Ref. 09-0080), JCPDS 2010 database.

The compressive strength of all prepared grafts was measured before and after *in vitro* ageing and subcutaneous implantation. Before testing, geometrical measurements of the graft cylinders were made in triplicate and the samples weighed. Samples were mounted on the testing machine (5544, Instron) so that the long axes of the cement cylinders were perpendicular to the lower anvil. A compressive force was then applied to the upper surface of the cylinders at a constant crosshead displacement rate of 1 mm/min until failure occurred. The applied load was measured using a 100 N load cell (5544, Instron). Mean compressive strength was determined from the average of 10 measurements.

After testing in compression, cement fragments were retrieved, weighed and dried in a vacuum desiccator at a temperature of 37 °C. The fragments were then ground to powder using a pestle and mortar. The true density of the powdered grafts was determined using a helium pycnometer (Accupyc 1330, Micromeritics). The volume of each sample was measured 10 times following 10 purges of the measurement chamber with helium. The relative porosity (bulk porosity) of the cements was calculated from apparent and true density measurements. The specific surface area (SSA) of the cement grafts in their solid cylindrical form was determined by using the Brunauer–Emmett–Teller (BET) method with helium adsorption–desorption (Tristar3000, Micromeritics).

Bioceramic microstructure was observed using scanning electron microscopy (SEM) (Hitachi S-4700 FE-SEM; Tokyo, Japan), at an accelerating voltage of 2 kV. Elemental composition of the bioceramics was assessed with energy dispersive X-ray (EDX) analysis using Oxford detector and INCA software (Oxford Instruments, Abingdon, UK). The pore size distribution of the prepared brushite and monetite cement grafts prior to *in vitro* and *in vivo* experiments was measured by using mercury intrusion porosimetry (9420, Micromeritics, Bedfordshire, UK).

2.3. In vitro ageing

After initial characterisation was complete, the graft cylinders were stored at 37 ± 1 °C and ∼100% relative humidity for 24 h. Brushite and the autoclaved and dry heat monetite grafts ($n = 3$) were immersed in PBS solutions (MP Biomedicals, LLC. Solon, OH. Cat No: 2810305), and also in bovine serum (Gibco, product code: 16170, New Zealand) containing sodium azide (Sigma–Aldrich) at a concentration of 0.1%. The graft cylinders were aged at a liquid to cement volume ratio (LCVR) of 60 as used by Grover et al. [20] for 7, 30 and 60 days at 37 ± 1 °C. Dynamic ageing protocols were achieved by refreshing the liquid every 24 h throughout the experiment to remove any dissolution products. To quantify the amount of mass loss over time, the graft cylinders were removed daily from the ageing medium and weighed. After periods of 7, 30 and 60 days of ageing, the grafts were removed from the solutions and tested in

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