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Fabrication and cytocompatibility of spherical magnesium ammonium phosphate granules

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ABSTRACT

Magnesium phosphate compounds, as for example struvite ($MgNH_4PO_4 \cdot 6H_2O$), have comparable characteristics to calcium phosphate bone substitutes, but degrade faster under physiological conditions. In the present work, we used a struvite forming calcium doped magnesium phosphate cement with the formulation $Ca_{0.75}Mg_{2.25}(PO_4)_2$ and an ammonium phosphate containing aqueous solution to produce round-shaped granules. For the fabrication of spherical granules, the cement paste was dispersed in a lipophilic liquid and stabilized by surfactants. The granules were characterized with respect to morphology, size distribution, phase composition, compressive strength, biocompatibility and solubility. In general, it was seen that small granules can hardly be produced by means of emulsification, when the raw material is a hydraulic paste, because long setting times promote coalescence of initially small unhardened cement droplets. Here, this problem was solved by using an aqueous solution containing both the secondary $(NH_4)_2HPO_4$ and primary ammonium phosphates $NH_4H_2PO_4$ to accelerate the setting reaction. This resulted in granules with 97 wt.% having a size in the range between 200 and 1000 μm. The novel solution composition doubled the compressive strength of the cement to 37 ± 5 MPa without affecting either the conversion to struvite or the cytocompatibility using human fetal osteoblasts.

1. Introduction

The use of autografts for the repair of bone defects is considered as "gold standard", but has several drawbacks such as a lack of availability and its donor site morbidity [1,2]. Alternative synthetic bone replacement materials are mostly based on calcium phosphate chemistry. Material approaches cover both the use of hydroxyapatite derived from natural resources (e.g. BioOss®) [3] as well as fully synthetic hydroxyapatites (HA) or tricalcium phosphates (TCP) prepared by sintering, precipitation or a cement setting reaction [4]. Depending on the chemical composition and crystal size, these materials are either stable (HA) or are able to slowly degrade in vivo (TCP) by active and passive mechanisms such that host autologous bone can grow into the bone defect [5]. Novel material approaches to enhance the degradation ability of bioceramics for bone replacement involve the use of magnesium phosphate compounds [6]. Such materials were found to be as cytocompatible as calcium phosphates, but offer a faster chemical degradation due to their higher solubility under physiological conditions [7]. Various magnesium phosphate compounds have been investigated in recent studies, e.g. struvite ($MgNH_4PO_4 \cdot 6H_2O$) [8–11], newberyite ($MgHPO_4 \cdot 3H_2O$) [12], amorphous magnesium phosphates [13] or magnesium doped calcium phosphates [7,14,15]. The formation of these compounds is often based on a cementitious reaction of either MgO or $Mg_3(PO_4)_2$ with aqueous sources of phosphate and ammonium salts. The solubility profiles of these cements may be altered by using biphasic calcium magnesium phosphate mixtures. This was already demonstrated by Vorndran et al. [11], who used compounds with the general formula $Ca_xMg_{(3-x)}(PO_4)_2$ allowing to adjust the setting properties as well as the mechanical performance and solubility. By using the formulation $Ca_{0.75}Mg_{2.25}(PO_4)_2$ together with a 3.5 M $(NH_4)_2HPO_4$ solution, a good biocompatibility in vitro [11] and in vivo [12], a high mechanical strength of ~80 MPa and an adequate setting time of 14 min were obtained [11].

In the present work, a cement based on $Ca_{0.75}Mg_{2.25}(PO_4)_2$ was used to fabricate pre-hardened cement granules since in dental surgery (e.g. sinus lift operation, socket preservation, filling of jaw cysts) the application of bone substitutes in granular form is a typical approach [16]. An easy way to generate granules on the basis of a hydraulic cement paste is the hardening of a cement monolith followed by

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mechanical grinding [3]. However, this technique leads to irregular sharp-edged granules that can promote irritation and inflammation in living tissues [16]. Another approach used a mixture of precipitated HA and chitosan, whereas the granules were hardened after starting the emulsification process by the addition of a cross-linker leading to granules with sizes in the range of 212 to 1000 μm [17]. However, in this case HA was not formed on the basis of a calcium phosphate cement (CPC) setting reaction but added as an inert filler to the biopolymer matrix prior to emulsification. Other techniques such as precipitation reactions would actually result in the fabrication of nanoscaled granules [18]. For example, Singh et al. [19] produced brushite nanoparticles by the combination of micro-emulsification with the process of precipitation.

In the present study, a more sophisticated approach was applied. It is based on dispersing the hydrophilic cement paste in a hydrophobic liquid (e.g. oil) by stirring while stabilizing the formed cement-oil dispersion using surfactants. The influence of different emulsification parameters (e.g. stirring energy, oil type, surfactant type and concentration, and aqueous solution composition) on the granules' properties (morphology, size distribution, degree of conversion, phase composition, cytocompatibility, and solubility) were tested. The main objective was the fabrication of spherical granules with clinically suitable diameters of 200 to 1000 μm.

2. Materials and methods

2.1. Fabrication of cement raw materials

$Ca_{0.75}Mg_{2.25}(PO_4)_2$ was prepared by sintering 1.5 mol $MgHPO_4 \cdot 3H_2O$ (Sigma Aldrich, Steinheim, Germany, Lot# BCBF1937V) with 0.5 mol $CaHPO_4$ (J.T. Baker, Griesheim, Germany, Lot# K06 587), 0.25 mol $CaCO_3$ (Merck, Darmstadt, Germany, Lot# A0074720 927) and 0.75 mol $Mg(OH)_2$ (Fluka, Steinheim, Germany, Lot# 10H040006) at 1100 °C for 5 h in a sintering furnace (Oyten Thermotechnic, Oyten, Germany). The product was crushed with a pestle and mortar, passed through a 355 μm sieve and milled in a planetary ball mill (Retsch, Haan, Germany) at 200 rpm with 500 mL agate jars, four agate balls (30 mm) and a load of 125 g powder per jar for up to 4 h. α-$Ca_3(PO_4)_2$ was synthesized by heating a mixture of 2 mol $CaHPO_4$ and 1 mol $CaCO_3$ to 1400 °C for 5 h. The product was crushed and sieved using a 355 μm sieve. 125 g of the powder was milled for 8 h under the above mentioned conditions, respectively.

2.2. Fabrication of spherical granules by emulsification process

Magnesium phosphate cement pastes were obtained by mixing 10 g $Ca_{0.75}Mg_{2.25}(PO_4)_2$ with a $(3.5-x)$ M $(NH_4)_2HPO_4$/x M $NH_4H_2PO_4$ solution (X = 0 to 2.0, Merck, Darmstadt, Germany, Lot# A0367907 314/A564126 728) in a powder to liquid ratio of 2.0 or 3.0 g/mL. The pH values of the different ammonium phosphate solutions were measured using a pH meter (inoLab pH Level 1, WTW, Weilheim, Germany). The cement paste was dispersed either in 300 mL Mygliol 812 (Caesar&Loretz, Hilden, Germany, Lot# 12356906) or safflower oil (Brökelmann&Co, Hamm, Germany), which was modified with 0.2 to 3.0% of either Tween 80 (Merck, Darmstadt, Germany, Lot# K37657061 746) or Span 80 (Sigma Aldrich, Steinheim, Germany, Lot# MKBD4327V) as surfactants. In case of using Brij 35 (Merck, Hohenbrunn, Germany, Lot# S42930762 744) as a surfactant, the compound was directly added to the cement paste as 3% aqueous solution. Dispersion was carried out in a 400 mL glass beaker using a mechanical stirrer RW16 basic (IKA-Werke, Staufen, Germany). Two differently sized half moon stirring blades (dimensions: 60 or 70 × 28 × 3 mm) and three different stirring rates (level 3, 4 or 5 corresponding to a rotational speed of 289, 427 and 556 rpm according to the manufacturer) were chosen for the emulsification process. After ~90 min, the cement was set and the granules were washed several times with water and acetone to remove oil and surfactant residues and dried in an oven at 37 °C. Some granules were additionally post-cured in 3.5 M $(NH_4)_2HPO_4$ solution for 24 h.

2.3. Characterization

2.3.1. Scanning electron microscopy

The granules' morphology was analyzed with scanning electron microscopy (DSM 940, Zeiss, Oberkochen, Germany) by using an acceleration voltage of 10 kV. Prior to this, a film of gold was deposited on the samples to avoid electrical charging.

2.3.2. Size distribution

The size distribution (wt.%) of the granules was determined by sieving them into fractions of > 2000, 1000, 710, 500, 355 and 200 μm.

2.3.3. X-ray diffraction analysis

The degree of conversion to struvite was determined by the addition of 20 wt.% of CaF_2 (Merck, Darmstadt, Germany, Lot# 308F652840) as an internal standard according to Alexander et al. [20]. X-ray diffraction patterns of the powder mixtures were recorded on a D5005 diffractometer (Siemens, Karlsruhe, Germany) with Cu-K_α radiation, a voltage of 40 kV and a current of 40 mA to analyze the qualitative and quantitative composition of the granules. Data were collected in a 2θ range from 10 to 40° with a step size of 0.02° and a scan rate of 1.5 s/step. The evaluation of the struvite integral peak intensity at 16.5° and the CaF_2 integral peak intensity at 28.3° revealed the degree of conversion.

2.3.4. Mechanical testing

For mechanical testing, the above mentioned cement pastes were transferred into silicon rubber molds (dimensions: 12 × 6 × 6 mm). These cuboids were hardened at 37 °C and 100% humidity for 24 h. The compressive strength measurements were performed using the universal testing machine Z010 (Zwick, Ulm, Germany) with a crosshead speed of 1 mm/min.

2.4. Biological testing

2.4.1. Cell culture

Human fetal osteoblast cell line hFOB (LGC Standards, Wesel, Germany) was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 1% penicillin and streptomycin, 0.3 mg/mL geneticin (G-418 sulfate) and 10% fetal calf serum (FCS, all from Invitrogen Life Technologies, Karlsruhe, Germany). The cells were incubated in a humidified 5% CO_2 incubator at 34 °C according to the supplier since they show a faster cell division at this temperature compared with body temperature.

2.4.2. Sample preparation

For cytocompatibility measurements, disk-shaped (dimensions: 15 × 2 mm) samples were made from the above mentioned cement paste. Hydroxyapatite (HA) cement disks were taken as control samples and prepared by mixing α-$Ca_3(PO_4)_2$ and a 2.5% Na_2HPO_4 (Merck, Darmstadt, Germany, Lot# F1485086 732) solution in a powder to liquid ratio of 3.0 g/mL. All the samples were hardened at 37 °C and 100% humidity for 24 h, washed in phosphate buffered saline for 14 days, disinfected in 70% ethanol and placed in quadruplicate in the wells of a 24-well plate (Nunc, Wiesbaden, Germany).

2.4.3. Cell number and proliferation

The cells were seeded on the cement samples with an initial density of 44,000 cells/cm^2 and on the polystyrene surfaces with 23,000 cells/cm^2, respectively. Because of their higher proliferation rate on polystyrene, less cells were seeded on this material. The cell culture medium was changed on days 3, 5 and 7, while after 4, 6 and 10 days of culture, the cells were both visually counted by means of a 0.4% Trypan blue