



The role of new zinc incorporated monetite cements on osteogenic differentiation of human mesenchymal stem cells



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ARTICLE INFO

Article history:

Received 14 December 2016

Received in revised form 13 April 2017

Accepted 15 April 2017

Available online 20 April 2017

Keywords:

Monetite cement

Zinc

Human mesenchymal stem cells

Zinc doped β -tricalcium phosphate particles

ABSTRACT

β -Tricalcium phosphate particles were sintered in the presence of different amounts (0–0.72 mol) of zinc oxide (ZnO) to prepare zinc doped β -TCP (Zn β -TCP) particles for further use in novel monetite (DCPA: CaHPO₄) zinc incorporated bone cements with osteogenic differentiation potential towards human mesenchymal stem cells (hMSCs). XRD analysis of zinc incorporated cements prepared with β -TCP reagent particles doped with different amount of ZnO (i.e. 0.03, 0.09 and 0.18 mol ZnO) revealed the presence of unreacted Zn β -TCP and monetite. Furthermore, it was shown that zinc ions preferentially occupied the β -TCP crystal lattice rather than the monetite one. Release experiments indicated a burst release of ions from the different fabricated cements during the first 24 h of immersion with zinc concentrations ranging between 85 and 100% of the total concentration released over a period of 21 days. Cell proliferation significantly increased ($P < 0.05$) on zinc incorporated monetite respect to control samples (Zinc-free cement) at 7 and 14 days post seeding. The expression of Runx-2 was significantly up regulated ($P < 0.05$) in the case of cells seeded on monetite prepared with β -TCP doped with 0.03 moles of ZnO. On the other hand, the cell mineralization as well as the expression of osteogenic marker genes ALP and OSC decreased significantly ($P < 0.05$) at 14 days post cell seeding. In conclusion, these results suggest that the zinc ions released from the cements during the first 24 h of culture played a critical role in regulating the osteogenic differentiation of hMSCs.

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1. Introduction

Autologous bone transplantation continues to remain the gold standard for treatment of bone loss due to different causes including osteomyelitis, impact fractures due to trauma and those resulting from bone weakened by osteoporosis. The advantage of autologous over synthetic bone graft materials resides in its osteogenic and osteoinductive properties [1]. However, bone is harvested generally from the patient's iliac crest and, as a consequence, requires a second operation at the site of tissue harvesting, which in turn may result in donor site injury and morbidity [2]. Furthermore, due to the limited amount of bone that can be harvested, autologous bone transplantation is not feasible for repairing large bone defects [3]. Therefore, a wide range of synthetic materials have been developed to overcome these drawbacks [4]. Among the different types, calcium phosphate grafts are considered the most favoured

alternative to autologous bone grafts due to their osteoconductive properties and ability to osseointegrate [5,6]. Calcium phosphates based materials can be used in bone defects in the form of granules, solid blocks or injectable cements. The latter are obtained by mixing a calcium phosphate powder (in a single composition or a mixture) with a liquid phase and during the mixing of the cement, reagent dissolution and re-precipitation takes place forming a CaP cement paste that solidifies in a set time, which is dependent on the chemical formulation [7]. Once implanted, a bone graft should allow cellular ingrowth, whilst resorbing for newly formed tissue and support new bone formation. Zinc ions have been shown to promote and sustain bone formation both in vitro and in vivo [8–10]. Hence, various studies have focused on the incorporation of zinc in the crystal lattice of bioceramics during high temperature synthesis or during the setting reaction of brushite cements [8, 11–15]. However, once implanted in the body, brushite tends to precipitate as insoluble hydroxyapatite (HA), slowing its replacement by bone [16]. On the other hand, anhydrous dicalcium phosphate (DCPA or monetite) is less soluble and appears not to transform to HA. Monetite is osteoconductive and resorbable in vivo [17,18], thus it forms an

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excellent candidate as a bone repair material or as a matrix for bone tissue engineering.

Earlier we reported a novel straightforward method for the production of porous monetite cements [19] and the same procedure was adopted to form zinc incorporated monetite cements in this study. The preparation of the cements was carried out in two steps:

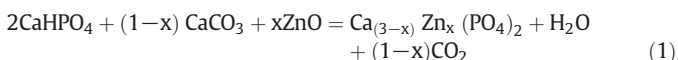
1. Sintering of Zn-doped β -TCP (Zn β -TCP) particles in the presence of different amounts (0–0.72 mol) of zinc oxide (ZnO).
2. Mixing of the homemade β -TCP or Zn β -TCP with commercially available monocalcium phosphate monohydrate (MCPM) particles in the presence of a liquid phase.

The dissolved zinc from the lattice of the doped Zn β -TCP during the setting is expected to enter within the monetite crystals, whilst unreacted Zn β -TCP particles in the set cement are expected to act as carrier of Zn ions in liquid medium. The Zn β -TCP particles were formed using a range of concentrations of zinc oxide. Amounts lower or equal to 0.09 mol yielded β -TCP particles with the zinc ions within the lattice. However, higher concentrations (from 0.18 up to 0.72 mol) led to the formation of a mixture of unreacted ZnO, Zn β -TCP and a zinc calcium phosphate phase (ZnCa₂(PO₄)₂). The aim of this work was the investigation of how the zinc changes the molecular microstructure of the phosphate cement and as the presence of Zinc in the dissolution medium of the phosphate has an influence on the cell biological response. Hence we investigated for the first time the proliferation and differentiation of hMSCs seeded on zinc incorporated resorbable monetite.

2. Experimental

2.1. β -TCP and Zn-doped β -TCP (Zn β -TCP) powders synthesis

β -TCP and Zn-doped β -TCP (Zn β -TCP) powders were prepared by a solid state reaction as shown in Eq. (1). Briefly, β -TCP powders doped with a molar percentage of Zn²⁺ ions ranging between 0 and 24% per mole of Ca²⁺ ions present in the β -TCP lattice were obtained by mixing zinc oxide, (ZnO, Sigma Aldrich, assay >99%) calcium phosphate dibasic (CaHPO₄, Sigma Aldrich, assay >98%) and calcium carbonate (CaCO₃, Sigma Aldrich, assay >99%) powders in a mortar for 5 min. Then, the whole mass was transferred to a steel mould and subjected to a pressure of 1 MPa for 2 min in order to obtain pellets with a volume of ca. 0.34 cm³. The resulting pellets were calcined in a furnace at a temperature of 1000 °C for 12 h in air. The different sintered powders were labelled in reference to the molar percentage of ZnO used (Table 1).



x = moles of Zn introduced in the powder mixture.

Table 1

The feed content of ZnO in moles is shown with the weight percentages. (%ZnO = percentage of moles of ZnO added to the powder mixture per mole of calcium ions present in the β -TCP lattice. x = moles of zinc introduced in the powder mixture).

ZnO %	ZnO Moles (X)	Powder	
0	0	Ca ₃ (PO ₄) ₂	→ β -TCP
1	0.03	Ca _{2.97} Zn _{0.03} (PO ₄) ₂	
3	0.09	Ca _{2.91} Zn _{0.09} (PO ₄) ₂	Zn β -TCP
6	0.18	Ca _{2.82} Zn _{0.18} (PO ₄) ₂	
9	0.27	Ca _{2.73} Zn _{0.27} (PO ₄) ₂	
12	0.36	Ca _{2.64} Zn _{0.36} (PO ₄) ₂	
18	0.54	Ca _{2.46} Zn _{0.54} (PO ₄) ₂	
24	0.72	Ca _{2.28} Zn _{0.72} (PO ₄) ₂	

2.2. Monetite cement preparation, setting time and SEM analysis

Monetite cements were prepared following the method described by Cama et al. [19] with the modifications, in which the cement precursors were Zn β -TCP particles. The setting times of the cements ($n = 3$) were evaluated using the Gilmore test according to ASTM C266-99 standard. The morphology of the sintered β -TCP powders and the microstructure of the monetite cements were observed by scanning electron microscopy (SEM-EDX, a Hitachi S-3500, Tokyo, Japan).

2.3. FTIR/ATR spectroscopy and X-ray powder diffractometry

FTIR/ATR measurements were performed by using a FT instrument (Perkin Elmer Spectrum One FT-IR Spectrometer, PerkinElmer Instrument, USA) with a resolution of 4 cm⁻¹. The crystalline compounds within the samples were determined using X-ray powder diffractometry (XRPD, PANalytical Corporation, Netherlands) with CoK α radiation. Samples were ground with an agate mortar and pestle, and the dry powders were mounted on zero-background silicon plates. Each sample was scanned between 3 and 70° 2 θ at a scan rate of 1° per minute. Identification was done using X'Pert High Score software (X'Pert-Pro MPD, Philips, Eindhoven, PanaLytical Corporation Netherlands) by comparing the diffraction pattern of the unknown compound against patterns for known compounds listed in the Powder Diffraction File (PDF 2000) database.

2.4. Diametral compressive strength and micro-CT analysis

The diametral strength of disc shaped specimens ($n = 12$, $d = 8.4$ mm, $h = 4.2$ mm) tested in compression was determined using a universal testing machine (Instron 5569A, Instron Corporation, USA) with a load cell of 500 N and a crosshead rate of 1 mm/min. The specimens after 24 h from the setting reaction were soaked for 24 h in distilled water and then still wet immediately tested. The porosity of the monetite cements were assessed using micro-CT imaging (SkyScan 1172 high-resolution micro-CT, Bruker micro-CT, Kontich, Belgium). All samples were scanned both at 4.96 μ m voxel resolutions using a source voltage of 100 kV and a current of 100 μ A with 0.5 mm aluminium filter to optimise the contrast. Samples were rotated 360° around their long axis and four absorption images were recorded every 0.400° of rotation. These raw images were then reconstructed with the standard Bruker micro-CT reconstruction software (NRecon, Version: 1.6.9.3, Bruker micro-CT., Kontich, Belgium) to serial coronal-oriented tomograms using 3D cone beam reconstruction algorithm. For reconstruction, beam hardening was set to 25% and ring artefact reduction to 12. The image analysis of the reconstructed axial bitmap images was performed using standard Bruker micro-CT software (CTan and CTvox). Cylindrical volume of interest (VOI) with a diameter of 4.0 mm and height of 3.5 mm was defined in the middle of each material. The greyscale threshold of binarised images was set from 50 to 255. Additional noise was removed by 'despeckling' function which removed all objects smaller than 1200 voxels that were not connected to the 3D object prior to further analysis and filtered with a Gaussian blur radius 2.0. The key 2D and 3D pore architecture parameters for each material both were then calculated in the CTan software. The 3D images were done in CTvox.

2.5. Calorimetry and ion release studies

A Calvet-type calorimeter (Setaram C80, SETARAM Instrumentation, Caluire, France) was used to measure the enthalpy of dissolution in water of ZnO and β -TCP powders obtained these latter by sintering in presence of 0, 3 and 6% ZnO, with a powder-liquid ratio P/L = 2:1. The heat changes during the setting of the cements prepared by using β -TCP with 0, 3 and 6% ZnO respectively were also

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