

Characterization of β -TCP, β -TCMP and BCMP Produced by Hydrolysis

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Abstract

Calcium Deficient Apatites (CDA's) were prepared by hydrolysis method. The CDA's were filtered, washed and calcined at 950°C for 11h. X-ray diffraction demonstrated that β -TCP was obtained after calcining Mg-free CDA and β -TCMP or BCMP were obtained after calcining Mg-substituted CDAs, depending on the Mg/Ca molar ratio of the CDA. Physicochemical characterization was also performed by FT-IR spectroscopy and inductive coupled plasma. Phenolphthalein test was performed, in order to investigate the presence of calcium oxide according to the French standard NF S 94-066. SEM images assessed the morphology of the compounds. Cell viability assay (MTT), calcium nodule formation and the expression of alkaline phosphatase (ALP), osteocalcin, TGF- β 1 and collagen were performed in MC3T3-E1 cell line. β -TCP, β -TCMP and BCMP obtained from hydrolysis method weren't toxic and promoted cell proliferation, showing potential value in bone tissue engineering.

Keywords: Tricalcium phosphate; Hydrolysis; Cell viability; Nodule formation; Bone markers

Introduction

Commercial synthetic calcium phosphate compounds used as bone graft materials include: Hydroxyapatite (HA); Tricalcium Phosphate (TCP); and Biphasic Calcium Phosphate (BCP) [1]. The last one consists of an intimate mixture of HA and β -TCP in varying HA/ β -TCP ratios. Mg substituted TCP (β -TCMP or Mg-TCP) as well as Mg-substituted BCP (BCMP or Mg-BCP) has been shown to be biocompatible and promote bone formation [2-5]. Therefore, both materials have potential to be applied as scaffolds for bone repair.

The overall objective of our study is to develop an Mg-substitute TCP and Mg-substitute BCP as potential materials for 3D printing. The specific aim of this work was to obtain TCP, Mg-substitute TCP and Mg-substitute BCP using the hydrolysis processing method and evaluate the toxicity and biocompatibility of these compounds.

Materials and Methods

CDA's were prepared by hydrolysis of commercial DCPD (dibasic calcium phosphate dehydrate) with or without a Mg source. The CDA's were filtered, washed and calcined at 950°C during 11h. The powders (green and calcined, with and without Mg) were characterized by: X-ray Diffraction (XRD) (Philips X'Pert X-Ray diffractometer), using a CuK α radiation at 45 KV and 15 mA (2 θ :20-40°), to evaluate their crystallinity and composition; and Fourier-transform infrared spectroscopy (FT-IR) (Nicolet Magna-IR 550 Spectrometer Series II), recorded in the range of 4000-400 cm⁻¹, to identify their absorption bands. The calcined powders were submitted to Inductively Coupled Plasma atomic emission spectroscopy (ICP) (Thermo Jarrell Ash, Trace Scan Advantage), where the specimen and standard solutions were pumped through argon plasma, which was excited by 2 kW/27.12 MHz radio frequency generator, to determine their calcium, phosphorus and magnesium concentrations. Phenolphthalein test was performed according to the French standard NF S 94-066 in order to detect the presence of calcium oxide, which is cytotoxic. The morphology of the green and calcined powders was observed under Scanning Electron Microscope (SEM) (Quanta FEG 250, FEI Company) working at 20 kV.

MTT assay was performed with MC3T3-E1 cell line (American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110, USA) to determine the cytotoxicity of the calcined powders.

In this assay, 100 μ L of 1x10⁶/ml cells were seeded in a 96 well plate and incubated for 24 h to allow adhesion. Then, cells were exposed to β -TCP, β -TCMP and BCMP extracts in different concentrations for 24h. 10 μ L of MTT were added per well 4hs before ending the treatment. When purple precipitates were clearly visible under microscope, 100 μ L of DMSO (Dimethyl Sulfoxide) were added to all wells to dissolve the formazan crystals. The absorbance of this colored solution was quantified by a spectrophotometer at a 570 nm wavelength. Calcium nodule formation was assessed as a function of osteogenic behavior of β -TCP, β -TCMP and BCMP compounds in MC3T3-E1 cell line. MC3T3-E1 cells were cultured up to 21 days, with 3 time points (1, 2 and 3 weeks). Mineralized nodules were detected by staining the cells with 0.1% Alizarin Red per 1 h and subsequently staining with 0.1% light green SF solution for 30min. The expression of alkaline phosphatase (ALP), osteocalcin, TGF- β 1 and collagen were performed in MC3T3-E1 cell line using SensoLyte[®] pNPP Alkaline Phosphatase Assay Kit (AnaSpec, Inc., Fremont, CA, USA), Mouse type I Collagen Detection Kit (Chondrex, Inc, Redmond, WA, USA), Mouse Osteocalcin EIA Kit (Biomedical Technologies, Inc, Stoughton, MA, USA) and Mouse TGF β 1 ELISA Kit (Insight Genomics, Fall Church, VA, USA), respectively, according to the manufacturer's instruction.

Results and Discussion

According to XRD spectra, β -TCP was obtained after calcining Mg-free CDA (Figure 1) and β TCMP or BCMP were obtained after calcining Mg-substituted CDAs, depending on the Mg/Ca molar ratio of the CDA (Figure 1). The Mg/Ca molar ratio obtained by ICP was 0.110 and 0.039 for the β -TCMP and BCMP samples, respectively. The FT-IR spectra of the calcined powders showed phosphate absorption bands at, approximately, 1125 and 1020 cm⁻¹, as well as at 605 and 555

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cm^{-1} in all materials, which are characteristic of tricalcium phosphates. The phenolphthalein test confirmed the absence of calcium oxide, which is cytotoxic. However, in this study, a first MTT assay showed a reduction in cell viability for β -TCP and β TCMP obtained from hydrolysis method, indicating toxicity for these compounds (Figure 2). After adjusting the processing method, a second MTT assay

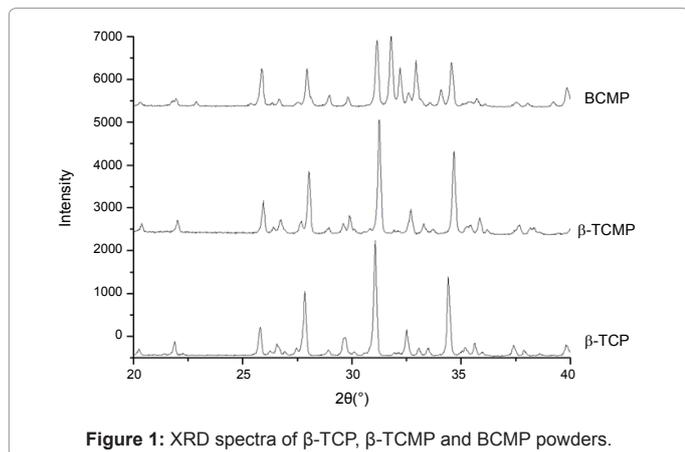


Figure 1: XRD spectra of β -TCP, β -TCMP and BCMP powders.

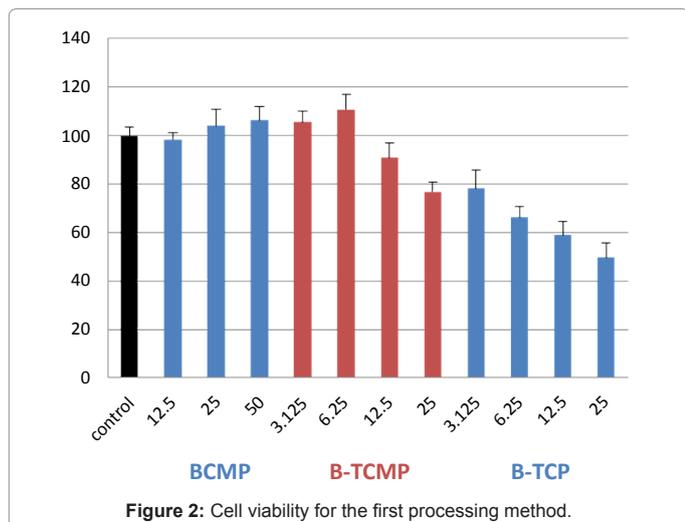


Figure 2: Cell viability for the first processing method.

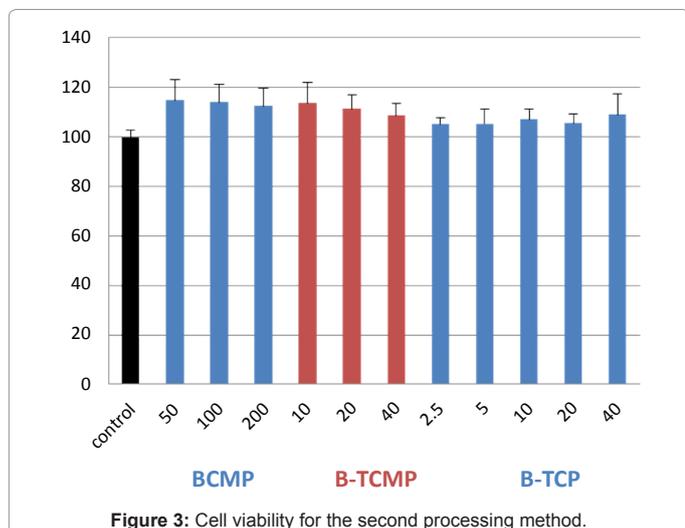


Figure 3: Cell viability for the second processing method.

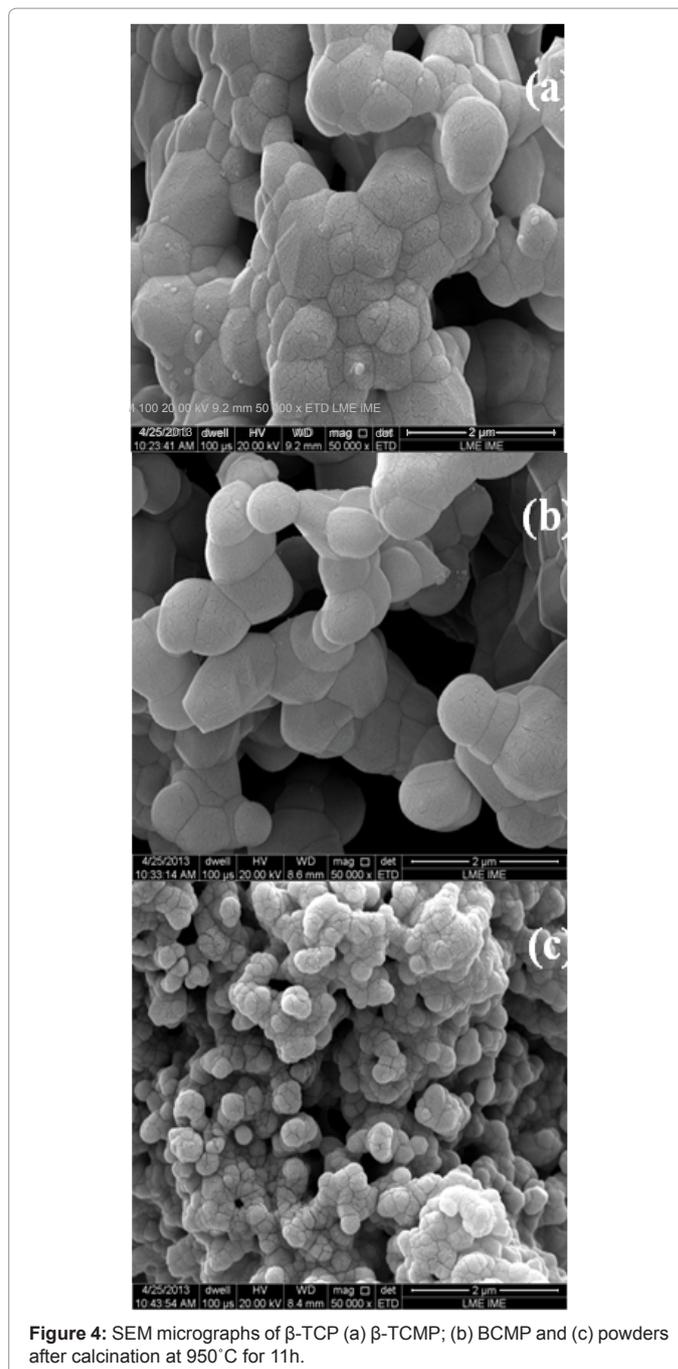


Figure 4: SEM micrographs of β -TCP (a) β -TCMP; (b) BCMP and (c) powders after calcination at 950 C for 11h.

indicated that the β -TCP, β -TCMP and BCMP obtained promoted cell proliferation, indicating non-toxicity (Figure 3). Calcium phosphate materials are generally considered biocompatible. Nevertheless, these results demonstrated that processing methods (e.g., washing) of the calcium phosphate preparations can affect the biocompatibility (toxicity) of the calcined products. Figure 4 shows SEM micrographs of the Calcium Phosphates (CaP) powders after calcination for 11 h. The morphology and size of calcined powders showed to be suitable for 3D printing purposes. In other words, after calcination, the morphology of the powder consisted of small dense round particles. According to Miranda et al. [6], small particle size, round and smooth surfaces are ideal features for starting powders used in inks for robotic deposition.

In this work, it was expected to obtain powders with particles size in the range below 50 μm , which was confirmed by SEM. Calcium nodule formation results indicated that the degradation products of the β -TCP, β -TCMP and BCMP are conducive to bone formation. The amount of mineralized nodules was higher in the β -TCP sample, followed by β -TCMP and BCMP samples. The same behavior was observed for bone formation markers (ALP and osteocalcin) and bone resorption markers (TGF- β 1 and collagen) activity in MC3T3 E1 cell line. This behavior can be explained for the fact that the presence of Mg ions retards the dissolution of CaP [7].

Conclusions

In this study, calcium phosphate materials as β -TCP, β -TCMP and BCMP were successfully produced via hydrolysis. The powders particle size and shape were suitable for robotic deposition. According to MTT assay, the compounds induced cell proliferation, which indicate non-toxicity. Calcium nodule formation and bone markers activity suggested that the materials present potential value in bone tissue engineering.

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