Current Applied Physics 14 (2014) 876–880

Contents lists available at ScienceDirect

Current Applied Physics

journal homepage: www.elsevier.com/locate/cap

Characterization and evaluation of cytotoxicity of biphasic calcium phosphate synthesized by a solid state reaction route

G.D. Webler [a], M.J.M. Zapata [b], L.C. Agra [c], E. Barreto [c], A.O.S. Silva [d], J.M. Hickmann [a,b], E.J.S. Fonseca [a,*]

[a] Institute of Physics, Federal University of Alagoas, Maceió 57072970, Brazil
[b] Institute of Physics, Federal University of Rio Grande do Sul, Porto Alegre 91501970, Brazil
[c] Laboratory of Cell Biology, Federal University of Alagoas, Maceió 57072970, Brazil
[d] Laboratory of Synthesis of Catalysts, Federal University of Alagoas, 57072970, Brazil

ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
6 March 2014
Accepted 30 March 2014
Available online 13 April 2014

Keywords:
Bioceramics
Hydroxyapatite
β-Tricalcium phosphate
Solid state reaction

ABSTRACT

Calcium phosphate compounds have been widely studied for biomedical applications because of their chemical and structural similarity to the mineral phase constituting bone and teeth. In this work, biphasic calcium phosphate ceramics (HAP/β-TCP), with tunable phase composition ratio, were synthesized by a solid state reaction process. The effect of varying the heat treatment temperature (700, 800, 900, 1000, and 1100 °C) on the formation of the ceramic materials and their related cytotoxicity were examined. The phase composition and morphology of the prepared ceramic powders were characterized by X-ray diffraction and scanning electron microscopy, and the functional groups were analyzed using Fourier transform infrared spectroscopy and Raman spectroscopy. Cell culture experiments, using murine macrophages, showed that the synthesized HAP/β-TCP materials did not exhibit cytotoxicity regardless of the doses assayed or the differences in composition ratio of HAP/β-TCP, suggesting the potential of HAP/β-TCP for biological applications.

1. Introduction

Calcium phosphate-based materials have received much attention as bone graft substitutes because of their composition similarity to that of bones, excellent biocompatibility, bioactivity, and osteoconduction properties [1–7]. Of the numerous calcium phosphate-based materials, hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) and β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$) are the most widely studied and clinically tested. Despite their comparable chemical composition, HAP and β-TCP display different biological and mechanical properties. For example, HAP is characterized as a low biodegradable ceramic [3,8] whereas β-TCP is considered as a resorbable bioceramic [1,3,4,6,7]. Resorbability—ability to be assimilated by living tissues—is an essential requirement for bone-like substitutes [6]. HAP also features poorer mechanical stability when compared with that of β-TCP [9–11].

Regardless, both materials (HAP and β-TCP) possess distinct properties suitable for specific biomedical applications. However, the combination of these materials, coined as biphasic calcium phosphate (BCP) materials, allows enhanced materials properties suitable for a larger range of applications. For instance, the bioreactivity and biodegradability of BCPs can be tuned accordingly to promote bone formation by adjusting their phase composition and porosity [2–5,8,12]. In addition, HAP matrices could benefit from increased mechanical stability by incorporating small amounts of β-TCP in their structure [9]. In vivo studies have also demonstrated the higher efficacy of BCP ceramics for bone repair when compared with that of pure HAP or β-TCP ceramics. Optimum HAP:β-TCP ratios of 70:30 and 60:40 have been reported [3,13].

HAP/β-TCP composite powders are common by-products obtained during the synthesis of pure HAP and β-TCP [4]. The two major processes for preparing HAP/β-TCP powders are the wet and the solid state reaction methods [1,14]. The former process involves the chemical reaction between the calcium and phosphate precursor solutions under controlled pH and temperature, producing a solid precipitate that is subsequently sintered [14]. Contrarily, the solid state reaction route generally involves the prolonged heat treatment of mechanically homogenized mixtures of calcium and phosphate compounds [14].

* Corresponding author. Tel./fax: +55 8232141424.
E-mail address: efonseca@optma.org (E.J.S. Fonseca).

http://dx.doi.org/10.1016/j.cap.2014.03.026
1567-1739/

In this study, biphasic calcium phosphate (HAP/β-TCP) materials were synthesized using a solid state reaction process at varying heat treatment temperatures. The influence of the varied synthesis temperatures on the formation of the crystalline ceramic materials and their respective cytotoxicity properties were investigated.

2. Experimental procedure

2.1. Synthesis

HAP/β-TCP composite materials were synthesized via a solid state reaction process, using calcium carbonate ($CaCO_3$, Sigma–Aldrich) and ammonium phosphate monobasic ($NH_4H_2PO_4$, Sigma–Aldrich) as metal precursor solids. Known quantities of the solid reagents were mixed (at molar ratio 1.67) and homogenized in an agate mortar. The mixture was then transferred to an alumina porcelain crucible and sintered at varying temperatures (700, 800, 900, 1000, and 1100 °C) in a high-temperature furnace (EDGCON 3P-3000) for 10 h at a heating rate of 10 °C/min.

2.2. Characterization

Thermal behavior (TG/DTA) analysis of raw materials were performed using a Shimadzu DTG-60H, in the 25–1100 °C temperature range, with a heating rate of 10 °C/min, in air.

The crystalline phase of the prepared materials was determined using X-ray powder diffraction (XRD) using Shimadzu Diffractometer (model 6000) with CuKα radiation ($\lambda = 1.5418$ Å). The samples were scanned from 10 to 70° at a scanning rate of 2°/min. The accelerating voltage and current employed were 40 kV and 30 mA, respectively. The relative phase content was quantitatively determined by the relative intensity ratio (RIR) method, using the following equation: $RIR = I_{\beta\text{-TCP}}/(I_{\beta\text{-TCP}} + I_{HAP})$ [3,5,14,15]. The intensities of the corresponding (2 1 1) and (0 2 10) principal diffraction peaks of HAP and β-TCP were used. The characteristic functional groups of the prepared materials were assessed on an infrared spectrometer (Prestige-21) equipped with a diffuse reflectance accessory (DRS-8000). For sample preparation, the powder sample (2 mg) was mixed with a non-absorbing standard (KBr, 200 mg) on a holder. The infrared spectra were recorded in the 4000–400 cm^{-1} region. Raman spectroscopy was used to characterize the vibrational properties of the prepared powders, which were loaded onto a glass slide for analysis. The data were collected in the range of 1200–400 cm^{-1} using a confocal Raman spectrometer (Nanonics Multiview 4000) equipped with a 532-nm laser. The morphology of the synthesized samples was observed using scanning electron microscopy (SEM, Shimadzu Super Scan SSX 550) under a high vacuum mode. Prior to analysis, the samples were coated with a thin layer of gold and platinum to improve the conductivity of the samples.

2.3. In vitro culture cell studies

MTT assay was used to evaluate the cytotoxic effect of HAP/β-TCP on macrophages according to the procedure by Mosmann [16]. Murine peritoneal macrophage (2.5×10^5) cells were treated with the various HAP/β-TCP materials, herein prepared, and at varying concentration doses ranging from 0.0001 to 1 μg/ml. The cells were cultured in an RPMI-1640 medium supplemented with 10% FBS for 24 h. Thereafter, the medium was replaced with fresh RPMI containing 5 mg/ml MTT. Following an incubation period (4 h) at 37 °C, the supernatant was discharged and dimethyl sulfoxide solution (DMSO, 150 μl/well) was added to each cultured plate. After incubation at room temperature for 15 min was complete, the absorbance of the solubilized MTT formazan product was spectrophotometrically measured at $\lambda_{max} = 540$ nm. Five individual wells were assayed per treatment and percentage viability was determined relative to the control sample as: (absorbance of treated cells/absorbance of untreated cells) × 100%.

3. Results and discussion

Fig. 1 shows the results from TG/DTA for the raw materials. For the $CaCO_3$, from TG curve, was possible to evaluate the weight loss of 43.86% in the temperature range 618 and 777 °C, approximately, due to the release of carbon dioxide giving rise to calcium oxide: $CaCO_3\,(s) + \text{heat} = CO_2\,(g) + CaO\,(s)$. This loss was confirmed by DTA curve, showing an endothermic peak at between 618 and 777 °C, which is characteristic of the decomposition of calcium carbonate. As for the $NH_4H_2PO_4$, the DTA curve shows an endothermic peak at 208 °C, this peak corresponds to the decomposition temperature of the crystal in accordance with the following equation:

$$2NH_4H_2PO_4(s) \rightarrow 2NH_3\,(g) + 3H_2O\,(g) + P_2O_5(s).$$

The equation above indicates a total weight loss of 38%, which can be observed in the TG curve until about the 580 °C. These losses are due to dissociation of the substance, evaporation of ammonia and water. Above 660 °C, P_2O_5 already formed starts evaporating, bringing the total weight loss to around 87.27%.

Fig. 2 shows the XRD patterns for the samples sintered at different temperatures (700, 800, 900, 1000, and 1100 °C) for 10 h.

The sample synthesized at 700 °C featured three crystalline phases: $CaCO_3$ (PDF #85-577), calcium oxide (CaO, PDF#77-2376), calcium phosphate (CaP_2O_6, PDF #11-39), and trace amounts of HAP (PDF #72-1243). As the synthesis temperature was increased to 800 °C, complete decomposition of $CaCO_3$ took place, as noted by the disappearance of the diffraction peaks, and formation of the BCP was instigated. Hence at synthesis temperatures ≥800 °C, only two phases were observed: HAP and β-TCP ($Ca_3(PO_4)_2$, PDF #70-2065).

The detailed solid state reaction sequence between the raw materials under heating during the synthesis can be understood by the thermal analysis, can be concluded as follows [17]:

$$10CaCO_3(s) + 6NH_4H_2PO_4(s) \xrightarrow{Heat} 3P_2O_5(s) + 10CaCO_3(s) + 6NH_3(g) + 9H_2O(g)$$

$$3P_2O_5(s) + 10CaCO_3(s) \xrightarrow{Heat} 3Ca_2P_2O_7(s) + 4CaCO_3(s) + 6CO_2(g)$$

$$3Ca_2P_2O_7(s) + 4CaCO_3(s) + H_2O(g) \xrightarrow{Heat} 3CaP_2O_6(s) + 3CaO(s) + 4CaCO_3(s) + H_2O(g)$$

$$3CaP_2O_6(s) + 3CaO(s) + 4CaCO_3(s) + H_2O(g) \xrightarrow{Heat} 3Ca_3(PO_4)_2(s) + CaO(s) + Ca_{10}(PO_4)_6(OH)_2(s)$$

By the XRD, is not possible to identify the CaO phase for samples synthesized at temperatures ≥800 °C, probably due to low concentration.

Based on the relative intensity of the principal diffraction peak of HAP at $2\theta = 31.74°$, the relative amount of HAP, in the sample sintered at 800 °C, is expected to be small when compared to that of the β-TCP phase. However, with increasing heat treatment temperatures, HAP became the more dominant phase as observed in Fig. 3, thereby suggesting that HAP is thermodynamically more stable than β-TCP [3].