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Three dimensional biphasic calcium phosphate nanocomposites for load bearing bioactive bone grafts

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ABSTRACT

Mimicking matrix mediated bio-mineralization process, three dimensional blocks of biphasic calcium phosphate (BCP, hydroxyapatite (HA) and β-tricalcium phosphate (TCP)) nanocomposites, having three different stoichiometries have been synthesized for possible application as load bearing synthetic bone graft or scaffolds. Biphasic blocks with three weight ratios of 20:80, 25:75 and 30:70 of HA and TCP respectively have been synthesized. Detailed structural and chemical characterization of the samples revealed a strong dependence of porosity and mechanical properties on the stoichiometry of biphasic blocks. Effect of physiological medium on the microstructure and mechanical properties of the three different blocks has also been studied. Bioactivity of the BCP block, exhibiting highest compressive strength in air as well as in physiological medium, has been evaluated through adhesion, proliferation and differentiation of mesenchymal stem cells using different markers.

1. Introduction

Use of calcium phosphates (Ca–P), a principal inorganic constituent of natural bone, as synthetic bone graft for bone tissue engineering has attracted a lot of research work over the last thirty years [1]. Different phases of calcium phosphates have been employed to fabricate microporous structures to accommodate bone tissue regeneration *in vitro* or *in vivo*. Among Ca–P family members, hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) are the two most extensively researched compounds. HA, being identical to inorganic mineral of natural bone is osteoconductive in nature but has a slow resorption rate in the physiological environment. Similarly β-TCP, having an optimum resorption rate, is characterized by poor mechanical stability [2]. Hence, selecting one of two as a bioactive (having osteoinductivity as well as osteoconductivity) bone graft has not been a successful approach for bone regeneration. The combination of a balanced ratio of HA and β-TCP, known as BCP materials is known to have desirable dissolution rate and optimum mechanical stability required for bioactive bone grafts [3–6]. Our group has already demonstrated synthesis of BCP nanopowders following a matrix mediated biomimetic synthesis process for Ca deficient hydroxyapatite and then its thermal decomposition to produce bioactive BCP nanopowder [7,8]. Variation of Ca/P ratio has been demonstrated to govern the stoichiometry of BCP nanopowders. It may be noted that bioceramic powders in general and nanopowders in particular have limitations in terms of its handling and application as bone grafts and hence three dimensional bioceramic geometries are required in different shapes and dimensions.

On the other hand, requirement to develop three dimensional bioactive synthetic bone grafts, needs an optimum combination of chemistry, mechanical stability and micro and macroporosity. Several groups have already reported the fabrication of 3D-load bearing porous biphasic calcium phosphate scaffolds with varying porosity and pore sizes by following different techniques including: replicas, sacrificial templates, direct foaming, gel casting with the addition of porogen/chemical binder and directional freezing to obtain macro/micro porous BCP composites [9–13]. In replica as well as sacrificial template methods, removal of the external template produces cracks and thus affects the mechanical properties of the scaffolds. Foaming methods often lead to the formation of isolated pores which lack of interconnection. Overcoming various limitations of different techniques, the present study reports a simple biomimetic process to produce 3D-calcium deficient polymer–HA nanocomposite having well defined external shape, followed by designed heat treatment for the removal of polymer and decomposition of Ca deficient HA into load bearing microporous BCP nanocomposites (compressive strength 6 MPa–27 MPa) with different stoichiometry (HA:TCP = 20:80, 25:75 and 30:70), keeping external geometry intact. Apart from detailed structural and chemical characterization, study also reports the performance of the synthesized nanocomposites in physiological medium and their osteogenic potential.

2. Experimental details

2.1. Materials

Calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$], diammonium hydrogen phosphate $(NH_4)_2HPO_4$, ammonia solution and polymer carboxymethyl cellulose (CMC) as sodium salt (degree of substitution:

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0.9) purchased from Merck, India for the *in-situ* synthesis of Ca-deficient hydroxyapatite (HA) nanocomposites. Deionized (DI) water was used in all the experiment.

2.2. Synthesis of three dimensional biphasic calcium phosphate nanocomposites blocks

3D-BCP blocks were synthesized by two steps: In first step calcium-deficient hydroxyapatite block ($Ca_{10-x}(HPO4)_x(PO4)_{6-x}(OH)_{2-x}$) was synthesized by a polymer mediated wet chemical method keeping the molar ratio of Ca/P in the range of 1.5–1.67. The polymer carboxymethyl cellulose (CMC) (5 wt.%) was used as matrix for the *in situ* synthesis of calcium deficient HA nanoparticles using three different molar ratios of Ca/P 1.62, 1.60 and 1.58 prior to the synthesis of BCP. Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$) and di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) were used as Ca- and P-source respectively. In a typical synthesis experiment, 22.19 g calcium nitrate tetrahydrate (0.94 M) was dissolved in 100 ml DI water and was made alkaline with ammonia (pH = 10). In another reaction vessel 0.5 g CMC was dissolved in 150 ml DI water to make a clear solution. 100 ml of the prepared Ca-salt solution was added to polymer solution slowly with stirring, kept for aging for a predetermined duration of 24 h at 30 °C and obtained desired characteristic of gel like solution. 9.33 g di-ammonium hydrogen phosphate (0.707 M) was dissolved in 100 ml DI water, made alkaline with ammonia. 100 ml of the prepared di-ammonium hydrogen phosphate solution was added gradually to the gel type solution of calcium in polymer matrix. Milky white coloration was observed instantaneously and the total volume of the slurry made up to 400 ml, which was allowed to age for a week at a temperature of 30 ± 2 °C. After a week the milky colored slurry was washed with DI water to neutralize. Neutralized slurry was transferred to a Teflon made beaker of size 500 ml and dried in an oven at 60 ± 2 °C for 72 h to obtain 3D-structures (10 g) of Ca-deficient hydroxyapatite (Fig. 1a) In the second step, 3D-Ca-deficient HA was heated at 900 °C for 2 h under controlled heating rate in a muffle furnace in air for thermal decomposition into 3D-BCP nanocomposites without destroying its external shape (Fig. 1b). Samples synthesized with Ca/P molar ratio of 1.62, 1.60 and 1.58 designated as BCP-1, BCP-2 and BCP-3 respectively. Experiments repeated three times to ensure the reproducibility of the phase formation.

2.3. Characterization

The crystalline inorganic phases of synthesized BCP nanocomposites were crystallographically characterized using X-ray diffractometry (XRD) (Bruker, D_8 Discover, Cu Kα radiation with λ = 0.154 nm, 40 kV, 40 mA) and for each sample XRD was taken thrice. Morphological characterization was performed using scanning electron microscopy (SEM). Shape and the size of biphasic nanoparticles have been characterized by transmission electron microscopy (TEM). The scratched BCP nanocomposites sample was dispersed into HPLC grade water using ultrasonication for 30 min. The suspended particles were lifted on a carbon-coated copper grid (# 200 mesh) with the help of forceps. The grid was dried on a filter paper under an infrared lamp, followed by inspection in the TEM. The physico-chemical characterization of BCP nanocomposites was performed using FTIR (JASCO-FTIR, Model 410) and infrared spectra were recorded in the range of 4000–400 cm^{-1} at resolution of 4 cm^{-1}. The samples were prepared as KBr pellet. The pore size distribution and porosity were determined by mercury intrusion porosimetry, (Model PASCAL 140/440, Thermo Electron). In this method, the sample was placed in a mercury filled dilatometer of known volume. It is then subjected to vacuum pressure up to 200 kPa under PASCAL-140 and to hydraulic pressure up to 250 MPa under PASCAL-440. As the mercury intrudes to pores under pressure, the capillary height in dilatometer lowers, which was a measure of mercury intruded to the pores under increasing pressure. As the pressure was released, the mercury was released out from the pores with sequential decrease in pressure. From this data, a plot of mercury penetrated and released with a change in pressure was plotted and the pore size distribution and porosity is computed from modeled data. Obtained results of the porosity measurements were correlated with microstructural features of the BCP nanocomposites by detailed scanning electron microscopy (SEM) studies. The compressive mechanical properties of the BCP nanocomposites of dimension 20 mm × 18 mm × 10 mm were tested using a Hounsfield Universal Testing machine-UTM, QMAT 3.75, ASTDM 695. The measurements of compressive strength of the BCP nanocomposites were made both in dry as well as in wet conditions. For wet condition measurements, samples were soaked in simulated body fluid (SBF) [14] for a maximum of four weeks. SBF was prepared with the ionic concentration nearly similar to human blood plasma. It is prepared according to procedure developed by Kokubo [14]. The appropriate quantities of reagents like NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, Na_2SO_4, and tris buffer were dissolved in 1 l of

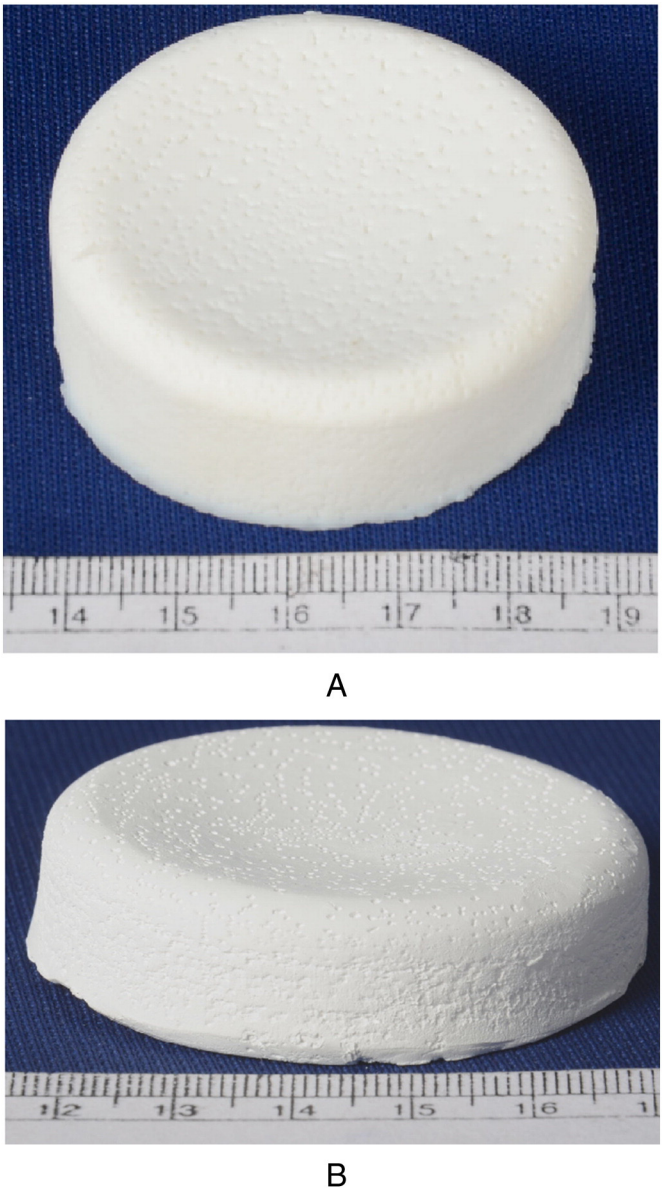

Fig. 1. a. As synthesized three dimensional Ca-deficient polymer-HA nanocomposite. b. Sintered three dimensional BCP nanocomposite.