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Avijit Guha^a & Suprabha Nayar^a

^a Biomaterials Lab , National Metallurgical Laboratory , Jamshedpur , India Accepted author version posted online: 22 Jun 2012.Published online: 25 Feb 2014.

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Low-Temperature Biomimetic Synthesis of β -Tricalcium Phosphate by Altering pH

Avijit Guha and Suprabha Nayar

Biomaterials Lab, National Metallurgical Laboratory, Jamshedpur, India

Biomimetic synthesis of β -tricalcium phosphate (β -TCP) in polyvinyl alcohol is done at a much lower temperature of 450°C compared with conventional synthesis. By varying the pH of the reaction in a patented process for hydroxyapatite synthesis, the authors synthesized β -TCP with controlled particle morphology at a very low temperature. The synthesized powder has been structurally characterized and cell studies with mesenchymal stem cells shows good adhesion.

Keywords β -Tricalcium phosphate, pH, polyvinyl alcohol

INTRODUCTION

Calcium phosphates (CaP) have excellent biocompatibility and have multiple applications in the field of tissue engineering, of which hydroxyapatite (HA) and β -TCP are very well studied.^[1,2] HA is the most stable phase, and osteoconductive and least soluble in the physiological environment.^[3] β -TCP, on the other hand, is one of the most resorbable phases and thus a more promising material in terms of faster healing.^[4,5] The variation in atomic Ca/P ratio in the synthesized calcium phosphate leads to differences in biodegradability and bioactivity.^[6-8] Although all calcium phosphates were initially considered as biocompatible but when used for clinical trials, they encounter many problems such as poor adhesion, biocompatibility, and weak mechanical strength.^[9,10] Reports suggest that incorporation of β -TCP particles into polyvinyl alcohol (PVA) matrix improves the biocompatibility, osteoconductivity, and bone bonding capacity of these materials,^[11] though there are many reports on the bonding mechanism of β -TCP with living bone, the issue is still debatable.[12,13]

There are many established techniques for β -TCP nanoparticles synthesis, which involves sintering at high temperatures, around 900–1200°C.^[14–21] When calcium-deficient HA Ca₉(HPO₄) (PO₄)₅(OH) is treated above 900°C it transforms into the β -TCP as shown by the following:

$$Ca_9(HPO_4)(PO_4)_5(OH) \xrightarrow{900-1200^\circ C} 3Ca_3(PO_4)_2 + H_2O$$

High thermal decomposition often leads to agglomeration and grain growth, thus causing a reduction in the surface area and porosity, and a subsequent loss of bioactivity. *In situ* synthesis of calcium phosphate nanoparticles in PVA matrix and its subsequent sintering at low temperature keeps the porosity and nanostructure intact. By varying the pH of the reaction, decomposition occurs at a much lower temperature, yielding macroporous β -TCP nanoparticles.

EXPERIMENTAL

Calcium nitrate [Ca(NO₃)₂.4H₂O] and diammonium hydrogen phosphate [(NH₄) ₂HPO₄] (both from Merck, India) were taken as Ca- and P-source, respectively. A 250 mL of 0.4M calcium salt solution was mixed with equal volume of aqueous solution of PVA (molecular weight 1 was 25000 and degree of hydrolysis was 85-89%, from Qualigens, India) containing 3.5 g polymer. The system was incubated for 24 h at 30°C. Stoichiometric 356 mL volume of 0.156M diammonium salt solution (pH > 8) was added to the previous mixture and mixed thoroughly. The entire mixture was adjusted to pH 7 with orthophosphoric acid (H₃PO₄) and aged for one week at 30° C; the precipitate obtained was washed with deionized water and oven dried at 80°C (S-1). The powder was calcined in a tubular furnace in air at a temperature 450°C for 2 h. Sample synthesized with Ca/P molar ratio of 1.50 has been designated as S-2. Experiments are repeated three times to ensure the reproducibility of phase formation.

Materials Characterization

To identify the phase of the synthesized materials, X-ray diffraction (XRD) was carried out using (Siemens, Germany, D500) using Cu K α radiation at 30 KV and 25 mA scanned for diffraction angles: $2\theta = -20^{\circ}$ to 80° at room temperature. To determine average crystallite size, Full width at half maxima (FWHM) was calculated according to Scherrer's equation with the shape factor K (0.9). Relative volume fractions of different crystalline phases formed were determined by the

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Address correspondence to Avijit Guha, Materials Lab, National Metallurgical Laboratory, Jamshedpur 831007, India. E-mail: bintuguha@indiatimes.com

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FIG. 1. XRD patterns of room temperature synthesized powder (S-1) and calcined sample (S-2).

area under the diffraction peaks of the respective phases. Size and shape of biphasic nanoparticles have been characterized using transmission electron microscopy (TEM; CM 200, CX Philips, Germany, at 160 KV JEOL 2100), the samples were ultrasonically dispersed in double distilled water and then one drop mounted on carbon coated copper grids. Thermogravimetric analysis of powders coupled with differential thermal analysis (DTA) was performed up to 1000°C (SDTQ600). A transformation from calcium phosphate to β -TCP was also ensured by Fourier transform spectroscopy (FTIR-410 JASCO, USA). The calcium phosphate molar ratio was analyzed by inductively coupled plasma-optical emission spectrometry analysis (ICP-OES). Samples were designated as (S-1) room temperature synthesized nanocomposite and (S-2) calcined sample.

0.2 µm

FIG. 3. High-resolution image shows growth in a preferred direction.

The powder was sterilized using UV and washed in Hanks's Balanced Salt Solution (HBSS). Cells were plated on cover slips and observed for 7-10 days. Cell adherence and growth was monitored every day and at the end of 10 days, cover slips were fixed with 2% glutaraldehyde and processed for scanning electron microscopy.

RESULTS AND DISCUSSION

Transmittance

It was observed that all the diffraction patterns matched with the standard calcium hydrogen phosphate and β -TCP (JCPDS file Nos. 65-2384 and 9-169) in both room temperature and treated samples (Figure 1). The diffraction patterns of the room temperature synthesized powder show the formation of a complex mixture comprising of calcium hydrogen phosphate and β -TCP, the 100% diffraction peak was identified and indexed as (112) corresponding to calcium phosphate along with a smaller

S-2

S-1

4000

FIG. 2. TEM micrograph of thermal treated sample.

Wavenumber cm FIG. 4. The FTIR spectra of room temperature and thermally treated samples.

2500

2000

1429)

1500

650

(873)

1000

(556)

500

(2917)

3000

(3444)

3500











FIG. 5. Thermal graph of as synthesized powder.

peak (102). The other peaks (125), (217), (128), (131), (1.1.15), (214), (128), 042), (404), (327), (120), and (2.0.20) correspond to β -TCP. After the sample was treated at 450°C for 2 h, the two peaks of calcium phosphate were not observed. All major diffraction peaks of the powder sample were identified and indexed as (214), (210), (2.1.10), (226), (1.0.16), (3.0.12), (238), and (2.0.20) peaks of β -TCP, with an average crystallite size of 40 nm.

TEM images confirmed the formation of nanosized crystalline β -TCP particles of needle shape and uniform size (24 nm length and 14 nm width; Figure 2). The lattice fringes were observed in micrographs that indicated the growth in a preferred direction (Figure 2).

The FTIR spectra of both samples are almost similar except the fact that absorbance at 3444 cm⁻¹ corresponding to hydroxyl groups in the thermally treated sample shows a decrease. The



FIG. 6. The cells adhered on the surface of the composite and proliferated.

absorbance band at 451 cm⁻¹ is assigned to doubly degenerate ν_2 O-P-O bending moment, ad the band at 556.72 cm⁻¹ is due to PO₄³⁻ vibration modes. The band at 873.96 cm⁻¹ is due to P-O(H) stretching in HPO₄⁻² group.^[22–25] Similarly, absorbance bands at 1026 and 1076 cm⁻¹ confirm the presence of P-O stretching mode. The absorbance band at 1429 cm⁻¹ correspond to a –CH₂ cm⁻¹ stretching band, confirming the presence of polymer in the synthesized samples. Another band at 2917 cm⁻¹ corresponds to a –CH- stretching mode. The band at 1632 cm⁻¹ also corresponds to –OH vibration mode (Figure 3).^[26–28]

The pH value of the reaction during synthesis is mainly controlled by H₃PO₄ and (NH₄) ₂HPO₄, responsible for the phase formation.^[29,30] Addition of H₃PO₄ solution to the mixture results in excess PO₄³⁻ ions, which dominates the pH drop from 10 to 7. At a lower pH, a large number of protonated phosphate ions (i.e., H₂PO₄¹⁻ and HPO₄²⁻) are present, which enters the crystal lattice and probably forms a Ca vacancy that is susceptible to heat treatment. On the other hand, the increase in the reaction pH promotes the dissociation of H₃PO₄ and (NH₄) ₂HPO₄ and enhances the formation of PO₄³⁻. Usually in the wet chemical synthesis high pH is required for the precipitation of stoichiometric and the thermally stable phase. Apatite crystals with calcium have a phosphate molar ratio of 1.63–1.33 that involves Ca and OH vacancies and can substitute some PO₄³⁻.

Thermal stability studies of sample shown in Figure 4. The thermograms of samples exhibit temperature difference in the peaks in the region of 200°C and 450°C.^[31,32] It is attributed to the removal of adherent water in the precipitated powder. Endothermic peaks in the range of 200–400°C are due to reversible water loss from the lattice of apatite structure caused by the rearrangement of oxygen atoms in the lattice and also it shows the decomposition of PVA molecules from the sample. The total weight loss of the sample was observed around (8%) at 450°C where the PVA decomposition as well as transformation occurred.

Mesenchymal cells (MSC) were isolated from the bone marrow; these cells were seeded onto the β -TCP nanocomposites. The culture was incubated for seven days in the CO₂ incubator with intermittent change of media. After seven days nanocomposites containing cells were examined under SEM. A large number of cells adhered on the surface of the composite and proliferated (Figure 5).

CONCLUSION

We have successfully synthesized β -TCP *in situ* in a PVA matrix at a low temperature. Addition of H₃PO₄ along with (NH₄)₂HPO₄ ions in the solution not only decreases the pH but also the excess HPO₄³⁻ ions cause an atomic imbalance in the reaction. The presence of high amount of HPO₄³⁻ ions in the solution decreases the Ca vacancies in the lattice, which renders the material unstable and forms calcium deficiency that is thermally unstable.

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