



Research paper

Novel continuous flow synthesis, characterization and antibacterial studies of nanoscale zinc substituted hydroxyapatite bioceramics



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ARTICLE INFO

Article history:

Received 1 June 2016

Received in revised form 14 July 2016

Accepted 25 July 2016

Available online 28 July 2016

Keywords:

Biomaterials

Ceramics

Calcium phosphate

Antibacterial property

X-ray diffraction

ABSTRACT

Calcium phosphates predominantly hydroxyapatite (HA) has extensively been used as bone substitute material and as coatings on metallic implants because of its high biocompatibility and bioactivity in bone mineralization. In order to enhance bioactivity of HA, hydroxyapatite has been substituted with divalent cations and anions. In this research, zinc substituted hydroxyapatite powder (<100 nm) was synthesized from aqueous solution of calcium nitrate tetrahydrate (with different concentration of Zn ions) and diammonium hydrogen phosphate in the presence of ammonium hydroxide solution through a novel continuous plastic flow synthesis (CPFS) at 70 °C in 5 min residence time at pH 11. The obtained powders were physically characterized using transmission electron microscopy (TEM), Scanning electron microscopy (SEM), BET surface area analysis, X-ray powder diffraction analysis (XRD), and FTIR and Raman spectroscopy. In addition, the particle size was evaluated by Dynamic light scattering (DLS). The chemical structural analysis of all as prepared samples was done using X-ray photoelectron spectroscopy (XPS).

The present study showed that all nanoscale particulates had rod like morphology (at low Zn concentration), which changed to semispherical morphology with increasing Zn contents. It was also investigated that zinc substituted hydroxyapatite gave better antibacterial protection than pure HA. Thus, the CPFS system facilitated rapid production of high surface area nanoscale crystals of hydroxyapatite modified by Zn ions with controlled particle properties (crystallinity, size, shape) at (near) ambient conditions.

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1. Introduction

Synthetic hydroxyapatite, HA, $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, is a bioactive bioceramic that is chemically analogous to the key mineral component of hard tissues (biological apatite). HA is greatly used as bone graft substitute in hard tissue replacement and as reinforcement material in biocomposites because of its high biocompatibility and osteoconductivity properties. It also has some drawbacks of slow growth rate and is also susceptible to bacterial activity during surgery [1–3]. Biological and physicochemical properties of synthetic HA can be enhanced using different cationic (Mg^{2+} , Mn^{2+} , Zn^{2+} , Na^+ , Sr^{2+}) and anionic (HPO_4^{2-} or CO_3^{2-}) substitutions. HA has ability to accommodate these substitute ions within its lattice. The structural surface and valency of hydroxyapatite is being

altered by the introduction of these ions and increased solubility stimulates the bone transformation more rapidly [4–6].

Synthesis of Zn substituted hydroxyapatite (ZnHA) is of major interest as zinc is a micronutrient, called essential trace element present in bone (100–200 mg/g) making 30% weight of skeletal muscles. The slow release of zinc from zinc substituted HA stimulates the growth of bone in vivo and in vitro environment [7]. This element has huge importance in medicine because of its function in more than 200 enzymes. Bioactivity of HA can be improved by using different level of Zn substitutions by replacing Ca^{2+} ions in the HA lattice [8]. It was observed that HA substituted with 5% Zn ion showed greater solubility than pure HA or HA substituted with low level of Zn. It also plays an important role in osteoblast cell proliferation and an inhibitory effect on osteoclastic resorption [9].

It must be noticed that one of the major problems associated with different bone implantations is the infection around the implants causing reconstruction surgery. Introducing antibacterial

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factors could reduce these infections by creating defense mechanisms around introducing biomaterials [10,11].

The antibacterial activity of HA could be enhanced by using different substitutions (Ag^+ , Zn^{2+} , Cu^{2+} and Ti^{4+}) into the HA for its long-term biomedical usage. In particular, Zn ion plays an important role in bone mineralization and pathological calcification because of its presence in biological apatite [12,13]. It has also been established that hydroxyapatite doped with Zn ions has a strong inhibition effect on the growth of various bacteria and fungi including *C. albicans*, *S. aureus*, *Streptococcus mutans* and *E. coli* [14]. Various zinc substituted hydroxyapatites have been synthesized using different reaction conditions and methods by replacing calcium with zinc. The most common methods are coprecipitation, solgel method, hydrothermal synthesis and combustion analysis [15].

To date, there have been no literature findings on direct and fast continuous flow synthesis at low temperature (70 °C) and atmospheric pressure for the synthesis of phase pure and ion substituted HA without stirring and further aging step. Most of the literature methods for the preparation of hydroxyapatite are either multiple steps or time consuming and require further steps of strong stirring and aging (from a few hours to couple of weeks) [16–20]. Thus, there is a demand to develop a single step, faster synthesis technique for the production of remarkably high surface area nanopowders with controlled particle properties.

The purpose of this study is to present a novel research technology with promising results for the synthesis of various high quality nanoscale biomaterials such as hydroxyapatite modified by Zn ions with better antibacterial activities.

2. Experimental

2.1. Reagents

Calcium nitrate tetrahydrate [$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 99%] Alpha and diammonium hydrogen phosphate [$(\text{NH}_4)_2\text{HPO}_4$, 98%] were purchased from Alpha Aesar, UK. Zinc nitrate hexahydrate [$\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 97%] was supplied by Sigma-Aldrich Chemical Company (Dorset, U.K.). Ammonium hydroxide solution (NH_4OH , 28%) was purchased by VWR International (UK). Deionised water was used throughout all experimentation.

2.2. Experimental procedure

In this process, 0.3 M diammonium hydrogen phosphate solution and 0.5 M calcium nitrate solutions containing different wt% of Zn ions (Zinc nitrate hexahydrate, $(\text{Ca} + \text{Zn})/\text{P}$ molar ratio = 1.67) ranging from 0 to 4 wt% were used. The pH of the solutions was kept at pH 11 by adding 15.0 ml of ammonium hydroxide to calcium nitrate (500 mL) and diammonium hydrogen phosphate solutions (500 ml), respectively. Both reagent solutions were pumped at 20 mL min^{-1} , to meet at a T-piece. This initial mixture were connected to 8 m long tubing surrounded by heating jacket. The flow rates used for calcium nitrate and diammonium hydrogen phosphate solutions for pump 1 and pump 2, respectively, were selected to give a total residence time of 5 min.

The suspension was collected in a beaker at the exit point. The precipitates were first filtered and washed followed by three washing cycles and then dried in oven at 90 °C for 12 h prior to further analysis. The oven dried filter cakes were then gently ground in a pestle and mortar to obtain fine powder. The samples were labelled as 0ZnHA (pure HA), 2ZnHA and 4ZnHA, respectively. The numbers in the sample IDs represented the expected zinc content (wt%). The product obtained was fine white powder with ~85% yield.

2.3. Characterization techniques

2.3.1. Powder X-ray diffraction

Bruker AXS D4 Endeavour diffractometer was used for XRD analysis of all samples. The data was analyzed in the 2θ range from 5 to 80° with a scanning step of 0.04° and a count time of 2 s/step using Cu-K α radiations ($\lambda = 1.5406 \text{ \AA}$). DIFFRAC^{plus} Eva software was used for the phase analysis of the data by spectral matching with standards patterns. The crystallite sizes were calculated by using Scherrer equation. The grid was dried prior to use in a double tilt holder of the TEM. Image J version 5.0 software was used for evaluating particle size.

$$L = 0.9\lambda/\beta \cos \theta$$

Where L = crystallite size, β = full-width half maximum of the p, λ = wavelength of X-rays (1.5406 nm for Cu-K α), θ = angle corresponding to the peak.

2.3.2. Transmission electron microscopy

Transmission electron microscopy was used to analyze the particle size and morphology. TEM was carried out on a JEOL 100CX microscope. A small amount of sample was dispersed in methanol solution and ultrasonicated for two minutes to yield a very dilute suspension. A few drops of the resulting suspension were then deposited on a carbon-coated copper grid (procured from Agar Scientific), which was used as a TEM specimen.

2.3.3. Chemical analysis

Chemical analysis of pure HA and Zn substituted HA were carried out using a Thermo Scientific K-Alpha X-ray photoelectron spectrometer (XPS). The vacuum analysis chamber pressure was $\sim 3 \times 10^{-8}$ Torr and a 128-channel position sensitive detector was used. The spectra were first collected using survey scans at 150 eV and thereafter; data for selected high-resolution regions (50 eV) was collected. The surface sensitivity, typically 40–100 Å makes this technique more ideal for measurements of elemental ratio as oxidation states. The XPS spectra were administered using CasaTM software.

2.3.4. Dynamic light scattering

Dynamic light scattering measurements were taken by using a Malvern Instruments Zetasizer functioned in backscatter (173°) mode. The sample slurry containing solid content of ~1% by volume was diluted with methanol. Ultra sonication was done for 10 min in order to make sample dispersion. This dispersed sample was then shifted to square cuvettes with a path length of 10 mm for measurements.

2.3.5. FTIR spectroscopy

Fourier Transform Infrared spectroscopy (FTIR) using a Nicolet 6700 FTIR (Thermo-Scientific, UK) was used for the identification of different functional groups in HA structure. The FTIR spectra were collected in mid-IR range 4000–400 cm^{-1} at 4 cm^{-1} resolutions, averaging 256 scans. Using Omnic 7TM software carried out data processing of the samples.

2.3.6. Raman spectroscopy

A Confocal Raman DXR Spectrometer (SP Thermo-Scientific) was used. The powder sample was deposited onto 316 L stainless steel block using a spatula. 316 L block was wiped clean first using distilled water then acetone prior to sample analysis. The data was collected using 780 nm lasers, 10 × Lens with the scan time of 90 s for each sample.

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