Advanced Powder Technology 25 (2014) 551–559

Contents lists available at ScienceDirect

Advanced Powder Technology

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

Original Research Paper

Influence of processing method on the properties of hydroxyapatite nanoparticles in the presence of different citrate ion concentrations

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ARTICLE INFO

Article history:
Received 12 September 2012
Received in revised form 29 July 2013
Accepted 10 September 2013
Available online 25 September 2013

Keywords:
Hydroxyapatite
Electron microscopy
Mechanical properties
Biomedical applications

ABSTRACT

The objective of this study was to investigate the effect of processing methods on the formation of ultra fine hydroxyapatite (HAp) nanoparticles in the presence of citrate ions and analyze their various physical properties. The addition of the citrate ions was found to reduce the size and prevent the agglomeration of HAp particles dramatically in the high gravity (HG) method compared to precipitation method. In precipitation method, the particle size reduced from 300 ± 70 nm to 90 ± 20 nm with the addition of citrate ions. In high gravity method, the particle size decreased more significantly from 80 ± 10 nm to 13 ± 5 nm with the addition of citrate ions. Furthermore, more uniform size distribution of nanoparticles was achieved in high gravity method. X-ray diffraction of nanoparticles prepared in both method exhibited slight shift of peaks to the higher angle with the addition of citric acid, indicating the incorporation of carbonate (CO_3) content in the HAp nanoparticles irrespective of the particle size. The mechanical properties of HWMPE matrix composite reinforced with nanoparticles was examined and this nanocomposite with nanoparticles prepared in high gravity method with the addition of citrate ions showed increased mechanical strength due to the considerable reduction in the particle size and higher uniformity of the particles. *In vitro* cellular analyses of the nanoparticle prepared in high gravity with the addition of citrate ions also displayed the most pronounced spreading of cell growth.

1. Introduction

There have been incessant attempts to develop bone substitute materials for biomedical applications. There is a growing need for research on bone restoration because of the ever-increasing incidences of various clinical bone diseases [1–3]. Hydroxyapatite (HAp), with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$, is a bioceramic which has an excellent biocompatibility and bioactivity because it comprises the main mineral component of teeth and bones and has a range of applications in various fields [4]. Bone can be considered as a nano-composite with nanometer-sized carbonated HAp as an inorganic constituent and collagen as an organic constituent. Hence, HAp is one of the most essential bioceramics for the biomedical applications, especially in orthopaedics and dental regeneration. However, the application of HAp in load bearing areas is quite limited because of the brittle nature of bulk HAp. The stiffness of real bone is mainly determined by the nano-scale apatite-like minerals [2–5], which are thought to be tougher than the coarser minerals. If the composition, morphology, and size of the synthetic bone minerals (apatite) can be controlled to have similarities to those of the natural bone to some extent, the mechanical stiffness, biocompatibility and osteoconductivity of synthetic apatite would be improved [5–8]. Owing to its small dimension and high specific surface area, the nanostructured apatite possesses some other special properties. The toughness of nano-structured apatite can also be enhanced by increasing strength and/or ductility as in other nanostructured ceramics and intermetallics [9] and also by adding some other elements [7]. The significant beneficial effect of nanostructured apatite material in comparison with usual micro-sized material was discussed by Webster et al. [10,11]. Additionally, several different methods have been employed to prepare desired HAp nanoparticles and reported in the literature [5,10–14]. However, it is not easy to obtain nano-phase HAp powders due to the agglomeration of the fine crystal powders using the normal synthetic methods.

This paper deals with the preparation of ultra fine HAp nanoparticles by using high gravity method and simple precipitation method in the presence of citrate ions as a dispersion reagent to prevent the agglomeration of HAp powders. High gravity method is a promising method for producing micro and nanoparticles in large scale [13,15–17]. In the previous studies, sub-micron and even nano particles, including $CaCO_3$, $SrCO_3$, and $Al(OH)_3$, were successfully produced

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0921-8831/$ - see front matter
http://dx.doi.org/10.1016/j.apt.2013.09.005

by a high gravity rotating packed bed (RPB) reactor without adding any surfactants to the reacting solution [15,16]. The main idea of the high gravity method is to intensify micromixing (mixing on the molecular scale) and mass transfer by higher rpm of RPB and therefore to enhance the reaction and control the process ideally [17]. Similar method called spinning disc processors have been successfully modeled and applied for the synthesis of $AgNO_3$ [18], $BaSO_4$ [19], and TiO_2 [20]. But that method is a two-step process where the reaction occurs at the surface of the spinning disc and is completed in the continuous stir tank reactor (CSTR). But the high gravity method used in the present study is a single step process where the complete reaction takes place in the RPB itself and the subsequent process can be continuously arranged. Yang et al. [21] reported the preparation of hydroxyapatite nanoparticles using high gravity precipitation combined with hydrothermal method which can be considered to be two step processes. We have reported the successful large scale production of hydroxyapatite nanospheres by the single step high gravity method [13]. In the present work we have tried to produce even more fine HA nanoparticle by adding citric acid as a chelating reagent in high gravity method so that we can take advantage of the combined effects and benefits of high gravity method and citric acid addition. Importantly, we have analyzed how these citrate ions influence the morphology and size and eventually improve the functionality of the material. The mechanical property of the material was analyzed by reinforcing the prepared material with high molecular weight polyethylene (HMWPE). Finally, the *in vitro* cellular analysis of the material was analyzed to confirm the improved biocompatibility of the material.

2. Experimental section

2.1. Materials

The starting materials used in the synthesis of hydroxyapatite, by both high gravity and precipitation methods, were analytical grade reagents calcium nitrate tertahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$). First, 1 M of $Ca(NO_3)_2{\cdot}4H_2O$ was dissolved in double distilled water. Then, 0.6 ml of $(NH_4)_2HPO_4$ was dissolved separately in deionized water and the pH of this solution was maintained at 9 using ammonium hydroxide (30%). Citric acid added nanocrystalline HAp powders were prepared by mixing 2 and 5 wt% citric acid into the calcium nitrate solution.

2.2. High gravity method

The detailed principle of the high gravity method was found elsewhere [13]. Briefly, prepared calcium and phosphate containing solutions were pumped through two different liquid inlets into the RPB (Fig. 1) of high gravity apparatus where both solutions were mixed and reacted with each other to form hydroxyapatite nanoparticles. The flow rates of calcium and phosphate solutions were controlled by using the liquid flow meter. In this study, the rotation speed (rpm) of the RPB and the flow rates of the solutions were maintained constantly. The rpm was set as 3000 and the flow rate of the liquid as 500 mL/min for all experiments. The mixed solution was flown back into the circulating tank through the solution outlet and was re-pumped into the RPB and mixed thoroughly with different rpm. This process was repeated two times in order to promote a through mixing and to enhance size uniformity of particles. Citric acid (2 and 5 wt%) added samples prepared in this method was labeled as HCHA2 and HCHA5 respectively).

2.3. Precipitation method

For precipitation method, the phosphate containing solution was added drop wise into the calcium containing solution and stirred vigorously for 12 h period. The as-synthesized HAp was afterwards filtered, washed with distilled water several times, and dried at 100 °C. Finally, the powders were calcined at 600 °C for 2 h. (Hereafter noted as CHA2 and CHA5 respectively).

Finally, obtained particles by both methods were washed several times with double distilled water and dried at 100 °C overnight. The dried powders were crushed with mortar and pestle and calcined at 600 °C for 1 h before further analysis.

2.4. In vitro cellular analysis

To investigate the cellular response to the citric acid added hydroxyapatite nanoparticles, CHO (CHO-K1, Korean Collection for Type Cultures) model animal cells were cultured on the nanoparticles. *In vitro* cellular assay was carried out by the following procedure. The powder samples were coated on 3 M adhesion tape. The prepared films were washed with PBS for 24 h and were then placed at the bottom of the wells of a multi-well tissue culture plate. After removing the PBS solution from the multiwall tissue culture plate by pipetting, the CHO cells (4×10^4 cm^{-2}) were seeded to the film surfaces. Ham's F-12 nutrient mixture (Gibco Laboratories) containing 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL gentamycin was used as the culture medium. The cells were cultured in an incubator at 37 °C under a 5% CO_2 atmosphere. At the end of each incubation period, the supernatant was withdrawn, and each well was washed with PBS and treated with trypsin (0.05% trypsin/0.02% ethylene-diamine-tetra-acetic acid, Gibco).

2.5. Characterization

The prepared samples were structurally characterized by X-ray diffraction (XRD) analysis using Cu Kα radiation (Rigaku X-ray diffractometer D/MAX-2200). The morphology, particle size, and size distribution of particles were investigated by a field emission scanning electron microscope (FESEM JEOL JSM-6500) at 10 kV after receiving a sputter coating of platinum for conduction. To gain further insight into the microstructures, transmission electron microscopic (TEM) investigations were performed using JEOL JEM-2100. Samples for TEM analysis were prepared by air-drying a drop of a sonicated suspension of the dried precipitate in ethanol onto copper grids. Selected area electron diffraction (SAED) pattern was also taken to examine the crystallinity of the samples. Particle size distribution analysis was carried out using HORIBA LB-550 particle size analyzer. Prepared HAp nanoparticles were thoroughly dispersed in ethanol for 30 min by ultrasonic agitator to remove the aggregated particles. Then the dispersed particles were analyzed by particle size analyzer. Zeta potential analysis was carried out using Colloidal Dynamics: Zeta Probe analyzer. The specific surface area of the prepared particles was measured by a nitrogen gas adsorption method using BET 201-APCW surface area analyzer. The Fourier transform infrared spectrum was obtained by using the KBr technique. The sample was blended with KBr in a 1:1 M ratio and pressed into a disk, and the measurements were done in the range from 400 to 4000 cm^{-1} at an interval of 4 cm^{-1} averaging 20 scans. The FTIR analysis was performed by using a SHIMADZU FTIR 8000S.

3. Results and discussion

3.1. X ray diffraction analysis

Figs. 2 and 3 shows the XRD patterns of the pristine and citric acid added samples prepared by precipitation and high gravity method respectively. Noticeably, the phase constituents of HAp powder have not been affected by the preparation method. It was observed that the addition of the citric acid introduces a