Materials and Design 87 (2015) 788–796

Contents lists available at ScienceDirect

Materials and Design

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

Effects of silicate and carbonate substitution on the properties of hydroxyapatite prepared by aqueous co-precipitation method

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ARTICLE INFO

Article history:
Received 13 February 2015
Received in revised form 27 July 2015
Accepted 16 August 2015
Available online 25 August 2015

Keywords:
Hydroxyapatite
Ionic-substituted hydroxyapatite
Characterization
In vitro evaluation
Cell response

ABSTRACT

This research was conducted to investigate the effects of silicate and carbonate substitutions in hydroxyapatite (HA) on phase retention, physical properties, and in vitro biological response. A wet chemical method was employed to synthesize silicate-substituted HA (Si–HA) and carbonate-substituted HA (CHA). It was shown that the presence of silicate and carbonate ions in the HA lattice increases the specific surface area and Ca/P molar ratio. In addition, the substitution also resulted in a reduction in the crystallinity of the HA powder and promoted better bioactivity of the sintered body. This was confirmed through in vitro cell culture experiment which revealed that the osteoblast cells adhered well on the surface of the ceramic and that ionic-substituted HA exhibited better cell performance than the control HA.

1. Introduction

Bioceramics made of calcium phosphates (CaPs) have been widely used as artificial bone substitute because of their excellent biocompatibility, bone bonding ability, and chemical composition similarity with natural bone [1,2]. Approximately 70 wt.% of bone is made of hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$), and therefore, HA has been intensively studied as a bone substitute material [3,4]. However, stoichiometric HA also does not dissolve over time but instead remain as a permanent fixture which can be susceptible to long-term failure [5]. Therefore, stoichiometric HA would not be suitable as bone substitute material which requires replacement by natural bone and hard tissues [3]. In addition, native bone differs from stoichiometric HA incorporated with other ions, such as carbonate, silicate, magnesium, and zinc. Reports confirm that the addition of ions that is present in native hard tissue into HA structure can lead to advantageous effects on biomaterial properties, such as structure order, solubility and dissolution rate. Therefore, a key target of biomaterial research is the preparation of a synthetic HA bone-substitute ceramic that mimics the chemical composition of hard tissue. Ionic substitution into HA (or CaPs) has been widely studied recently [6–9].

Bone apatite contains approximately 3 wt.% to 8 wt.% carbonate ion, and hence, bone apatite has been referred to as carbonate apatite (CHA) [5]. The incorporation of carbonate ions is known to pose a considerable impact on the crystal lattice of apatite structure and on the mineralization process [10]. Introduction of carbonate ions in HA promotes dissolution and could enhance the osteointegration rate [11–13]. In addition, CHA can be resorbed by osteoclasts and be replaced with new bone [14]. Therefore, carbonate-substituted HA or CHA is a prospective candidate for bone-substitute material.

Apart from carbonate, silicon is a trace element found in bone with a specific metabolic role connected to bone growth, especially during the initial formation stages [15,16]. Silicate substitution at a small concentration simulated the activation of seven families of genes in osteoblast [17], thus increasing osteoblast proliferation, differentiation, and bone extracellular matrix production. Although no study has linked the improved biological performance of Si-substituted CaPs to Si release, and no evidence of silicon released rate from Si–HA in vivo is reported, the presence of Si in the HA structure is widely accepted to cause chemical or topographical changes that would eventually lead to a change in biological responses [18]. In addition, Si substitution promotes ion release that is essential for biological process [19].

The solubility of ionic substituted HA was observed to be higher than that of pure HA. This difference can be attributed to an increase in structural order, which is due to the presence of foreign ions [20–22] in the HA structure. However, only a few studies have investigated the ion release in synthetic fluids [22]. Although research on silicate or carbonate-substituted HA have been reported in the literatures [7,15,23], the actual effect of the ion substitutions have not been well

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http://dx.doi.org/10.1016/j.matdes.2015.08.069
0264-1275/

understood. In addition, a systematic comparison of the physiochemical properties of SiO_4^{4-} and CO_3^{2-}substituted HA has not been carried out. Also, the interaction between biological cells and ionic substitution (SiO_4^{4-} and CO_3^{2-}) of HA synthesized using similar precipitation conditions have not been studied. Therefore, the purpose of the present work is to investigate the effect of ion substitutions of $SiO_4^{4-}$and CO_3^{2-} into the HA structure on the phase retention, physiochemical properties, and early in vitro biological response. The paper reports the preparation and characterization of CHA and silicate-substituted HA (Si–HA) by a precipitation method. In this work, the prepared powders were subjected to heat-treatment at various temperatures (800 and 1200 °C). The powder analysis was carried out by means of powder X-ray diffraction (XRD), transmission electron microscope (TEM), fourier transform infrared (FTIR), X-ray fluorescence (XRF), inductively coupled plasma atomic emission spectroscopy (ICP/AES), specific surface area by means of the BET (Brunauer,Emmet and Teller) while the morphology of heat-treated compact was observed under scanning electron microscope (SEM). In addition, the biocompatibility nature of these materials in vitro with MC3T3-E1 osteoblast-like cell was also evaluated.

2. Experimental procedures

2.1. Powder synthesis

Preparative conditions of alkalinity pH = 9.4 ± 0.1 and reaction temperature of 40 °C ± 1 °C were used to synthesize pure HA and HA substituted with SiO_4^{4-}(1.6 wt.% Si) and CO_3^{2-}(8 wt.% to 10 wt.% carbonate content) by aqueous precipitation. The reaction was allowed to proceed in a closed system of a reaction flask placed in a heating mantle and under overhead stirring at 400 rpm. Pure HA powder, Si–HA powder, and CHA powder were synthesized using calcium hydroxide [$Ca(OH)_2$, 96% purity, Fluka], phosphoric acid (H_3PO_4, Merck), silicon tetraacetate [$Si(COOCH_3)_4$, 98% purity, Sigma-Aldrich], and CO_2 gas as Ca, P, Si, and carbonate sources. The Ca/P molar ratio of the precursors was based on 1.67 for the synthesis of the HA, CHA and Si-HA powders. To obtain the highest degree of carbonation and a B-type CHA (carbonate ion replace for phosphate ion) precipitation with respect to A-type (carbonate ion replace for hydroxyl ion), the CO_2 gas flow was set at 1 bubble/2 s as outlet flux [12].

The PO_4^{3-}solution was added drop-wise (~1.25 mL/min) to Ca^{2+} solution under continuous stirring to obtain pure HA. For the synthesis of Si–HA, a preset amount of $Si(COOCH_3)_4$ was first dissolved in the Ca^{2+}solution prior to the addition of PO_4^{3-} solution. To form CHA, the PO_4^{3-} solution was added to Ca^{2+} solution, while CO_2 gas (40 mL/min) was passed through the reaction flask. The pH was maintained at 9.4 ± 0.1 by a pH meter by adding small amount of ammonium hydroxide (NH_4OH 29%, J.T. Baker, USA). The final solution was continuously stirred for 2 h at 40 °C and left to settle for 1 day. The precipitate was then separated by filtering, washing with deionized water, and drying at 70 °C for 24 h. Finally, the precipitate was ground into a fine powder prior to uniaxial compacting at 60 MPa into disc samples (11 mm diameter × 3 mm thickness). The HA and Si–HA green disks were sintered in air at 1200 °C for 2 h, whereas the CHA compact was sintered at 800 °C for 2 h in CO_2 gas (40 mL/min) to prevent carbonate loss. The heating and cooling rates were set at 5 °C/min.

2.2. In-vitro experiment

2.2.1. Simulated body fluid (SBF) test

In vitro experiments were performed on substrates of the heat-treated pure HA, Si–HA, and CHA samples (25 ml SBF, sample size ~10 mm × 2.8 mm). Ion release evaluation was performed in triplicate by immersing the compacts in a SBF solution at 36.5 °C for 1, 3 and 7 days. The SBF solution was prepared according to the procedure described by Kokubo et al. [25]. After the predetermined soaking time, the samples were removed, the ion concentrations of liquid media were analyzed by ICP, and pH was measured by a pH meter.

2.2.2. Cell culture test: cell morphology and proliferation

Prior to cell culture, all heat-treated samples were sterilized by heating in a vacuum oven at 120 °C for 3 h. MC3T3-E1 osteoblast-like cells (Riken Cell Bank, Tokyo, Japan) were cultured in L-glutamine containing alpha-minimum essential media (α-MEM, GIBCO/Invitrogen, Grand Island, NY, USA) supplemented with 10 vol.% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), and 1 vol.% penicillin (10,000 units) and streptomycin (10 mg/mL). Cells were maintained at 37 °C under 5% CO_2 in a humidified atmosphere. The medium was changed every 2 days. At confluence, adherent cells were passaged and harvested using 0.25% trypsin–EDTA (Trypsin; GIBCO, Invitrogen™, NY. USA). Cells at passage 2 were used for culture on the sample surface with a cell density of 2×10^4 cell/mL. Each experiment was performed in quadruple ($n = 4$) for each sample group. Cell morphology and attachment ability were observed after 4 h, 1 day and 3 days of culture duration. Cell proliferation was evaluated after 2, 4, 6 and 8 days of culture using Alamar Blue dye test (AlamarBlue™; BioSource International Inc., Camarillo, CA, USA). At the end of each time point, the culture medium was replaced with culture medium containing 10% Alamar Blue reagent. Specimens with cells were then further incubated for 4 h. The resulting 200 μL solution was obtained from all wells, placed in a 96-well clear bottom plate, and the fluorescence was measured on a plate reader (Tecan Infinite M200, Austria GmbH, Grödig, Austria) using an excitation and emission wavelength of 520 and 590 nm, respectively.

2.2.3. Osteoblastic differentiation: alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured by LabAssay™ ALP (Wako, Japan) using p-nitrophenylphosphate as the substrate following instruction of the manufacturer. Cells were seeded onto specimens at an initial density of 4×10^4 cells/mL. After 7, 14 and 21 days, cells attached on the specimens were rinsed twice with PBS and lysed with cell lysis buffer M (Wako, Japan) for 30 min. After centrifugation, 20 μL cell lysate supernatant was incubated with the assay buffer at 37 °C for 15 min and the absorbance was spectrophotometrically measured at 405 nm. Total protein content was measured using Bio-Rad Protein Assay (Pierce Chemical Co., Illinois, USA). 5 μL of the cell lysate supernatant was added to 100 μL assay buffer and shake for 1 min, and then spectrophotometrically measured at 595 nm. ALP activity was normalized by total protein content and expressed as unit/μg protein.

2.2.4. Mineralization assay

Cells were seeded at an initial density of 4×10^4 cells/mL. After 14 days of culture, specimens were washed twice with PBS and the cells were fixed with 99.5% methanol for 30 min at room temperature. After rinsing with distilled water, specimens were stained by Calcified Nodule Staining Kit (Cosmo Bio Co., Ltd) solution composed of Alizarin Red S and Chromogenic Substrate for 5 min. After removing excess dye by substrate-containing buffer, the images of stained specimens were observed using an optical microscope (LG-PS2, Olympus, Tokyo, Japan). For the calcium quantification, after 14 and 21 days of culture, specimens were washed twice with PBS and ultrapure water. 1 ml of 0.1 mol/L HCl was subsequently added and the plate was subjected to vibration for 12 h at room temperature. The suspension was centrifuged at 12,000 rpm for 5 min. Calcium content of the supernatant was measured using a calcium C-test Wako kit (Wako, Japan) at an absorbance wavelength of 610 nm.

2.3. Material characterization

The phases present in the synthesized powders were examined by X-ray diffraction (XRD; D5000 Siemens) using CuKα radiation (λ =