Materials Science and Engineering C 62 (2016) 183–189

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Hydroxyapatite from fish scale for potential use as bone scaffold or regenerative material

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form 18 January 2016
Accepted 20 January 2016
Available online 22 January 2016

Keywords:
Hydroxyapatite
Fish scale
Bone tissue engineering
Natural resource
Bone scaffolds

ABSTRACT

The present paper studies the physico-chemical, bioactivity and biological properties of hydroxyapatite (HA) which is derived from fish scale (FS) (FSHA) and compares them with those of synthesized HA (sHA) obtained by co-precipitation from chemical solution as a standard. The analysis shows that the FSHA is composed of flat-plate nanocrystal with a narrow width size of about 15–20 nm and having a range of 100 nm in length and that the calcium phosphate ratio (Ca/P) is 2.01 (Ca-rich CaP). Whereas, synthesized HA consists of sub-micron HA particle having a Ca/P ratio of 1.65. Bioactivity test shows that the FSHA forms more new apatite than does the sHA after being incubated in simulated body fluid (SBF) for 7 days. Moreover, the biocompatibility study shows a higher osteoblast like cell adhesion on the FSHA surface than on the sHA substrate after 3 days of culturing. Our results also show the shape of the osteoblast cells on the FSHA changes from being a rounded shape to being a flattened shape reflecting its spreading behavior on this surface. MTT assay and ALP analysis show significant increases in the proliferation and activity of osteoblasts over the FSHA scaffold after 5 days of culturing as compared to those covering the sHA substrates. These results confirm that the bio-materials derived from fish scale (FSHA) are biologically better than the chemically synthesized HA and have the potential for use as a bone scaffold or as regenerative materials.

1. Introduction

Trauma, certain diseases, and physical force may lead to fracture of bone and may result in dangerous complications, especially in the elderly. In recent years, biomaterials based on calcium phosphate especially hydroxyapatite (HA) have been widely used to repair bone fractures and other defects because of their osteoinductive and osteoconductive properties [1–4]. Other features which make hydroxyapatite attractive for use as bone replacement are that it is the major component of bones and hard tissues, is non-toxic and non-immunogenic, has the mechanical strength and has the surface properties desirable for bone tissue regeneration [2]. For these reasons, it has been widely used in orthopedics and dental applications [3]. Most of the studies on the use of HA as bone substituted materials employ chemically synthesized hydroxyapatite (sHA) (from solutions containing calcium and phosphate ions) and the nanostructures with different morphologies including nanorods, nanobundles and nanoparticles have been prepared *via* a simple precipitation method [5–9]. However, sHA is different from the natural hydroxyapatite in human bone in terms of several of their physicochemical properties such as their strength and chemical composition [10,11], which leads to sHA having lower biological activity than natural hydroxyapatite. Based on the premise that if the properties (such as chemical composition and structure) of biological HA are preserved at the nanoscale level, beneficial effects due to the HA properties could be achieved and the biomaterial would be better accepted by the living organs (and be biologically safe). The stratagem of extracting them from natural sources has been previously investigated. In recent year, various natural materials, such as fish bones, fish scale and mineral materials have been used to produce HA [12–20]. Chemical analysis reveals that these products are abundant sources of calcium phosphate and could be used as an economic source for HA synthesis. In addition, these materials would be seen as being by-products (biowaste) from the food industry. The conversion of such biowaste would be considered to be one of the benefits for a solid waste management, reduced environmental impact.

To study the natural derived HA products for use as bone substituted materials, Milovac et al. and Beom-Su et al. [12,13] used cuttlefish-bone-derived hydroxyapatite (CB-HA). They compared the properties of the CB-HA derived materials with those of the synthetic hydroxyapatite.

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http://dx.doi.org/10.1016/j.msec.2016.01.051
0928-4931/

Their results showed that CB-HA was more non-toxic and exhibited greater cell adhesion, proliferation and differentiation when compared to those of synthetic HA. HA derived from the bones of sword fish (*Xiphias gladius*) and tuna (*Thunnus thynnus*) revealed that these materials were non-toxic and could be a promising new bioactive material [14]. Research on producing HA from waste shells were investigated by Shavandi et al. [15] and Tâmâsan et al. [16]. Based on their results, it was proposed that these HAs had the potential to be materials for use in bone tissue engineering applications. Fish scales are another source of natural materials which could be used to synthesize HA. The preparation of HA from fresh water fish scale (*Labeo rohita*) and biocompatibility was done by Modal et al. [19]. They found that the cells grown on the scaffold made with these types of HA materials promoted cellular attachment and proliferation. However, the conversion of such raw materials into HA, an alkaline heat treatment method (thermal treatment at 800 °C with basic $CaCl_2$) had to be employed to obtain the HA ceramics. Such a thermal treatment led to a HA/β-TCP biphasic material and could change the physicochemical and biological activities of the original materials. In this paper, we obtained the HA material from the alkaline hydrolysis fresh *Probarbus jullieni* scales (FS) without the high temperature treatment. These FSHAs were characterized by several analytical techniques to determine their composition and microstructural features and tested for their biological activity. These properties were then compared to the properties of the synthetic HA grown from solution.

2. Experimental

2.1. Materials

Chemicals used in this experiment are $Ca(NO_3)_2 \cdot 4H_2O$ (calcium nitrate) (Fluka Chemika, Switzerland), $(NH_4)_2HPO_4$ (diammonium hydrogen phosphate) (Fisher Scientific, UK), sodium hydroxide (NaOH) (Merck, Germany), NH_4OH (Merck, Germany) and hydrochloric acid (HCl) (Mallinckrodt chemicals, USA). They were used without further purification.

2.2. Extraction of hydroxyapatite from fish scale (FSHA)

The scales were taken from the fresh water fish (*P. jullieni*) raised in a fish farm located in Ratchaburi Province, Thailand. The details of the preparations being; the fresh *P. jullieni* scales (FS) were first rinsed thoroughly with water to remove any grease. The adhering tissue was manually scraped. Thereafter, the scales were soaked and stirred in 4% hydrochloric acid (HCl) solution for 15 min at room temperature for deproteinization. Afterwards the solution was neutralized by sodium hydroxide (NaOH) treatment to obtain hydroxyapatite-rich slurry and was subsequently filtered through a filtered-pressed machine having a 500 μm diameter pore membrane. The resulting obtained FSHA cake was sealed in a plastic bag and boiled in distilled water at 100 °C for 30 min to deactivate the enzymes, and kept at −20 °C for further used. Before characterization, the FSHA was dried at 60 °C in a hot air oven.

2.3. Synthesis of hydroxyapatite (sHA)

Synthesis of calcium phosphate of hydroxyapatite (sHA) powder was done by an aqueous precipitation reaction. Briefly, $Ca(NO_3)_2 \cdot 4H_2O$ (0.40 M) and $(NH_4)_2HPO_4$ (0.24 M) solutions were gradually mixed together at room temperature, with the simultaneous adjustment of pH solution to 11 with NH_4OH. The supernatant solutions were then removed from the powder. The resulting powders were washed with de-ionized water until the pH was reduced to about 7 and then freeze-dried. Before performing the physicochemical and biological measurements, samples of the powders were heated to 500 °C for 2 h in an ambient air atmosphere to thoroughly dry them.

2.4. Characterization of FSHA and sHA

The crystal structures of the composite pellets were determined by powder X-ray diffraction (XRD) (Bruker diffractometer, Model D8 Advance) using the CuK_α radiation and operating at 40 kV and 40 mA current. The angles scanned were from $2\theta = 10°$ to 60° at a scanning speed of 1 incremental step of 0.037° per second. For the FT-IR absorption measurements, the powders were mixed with KBr and pressed into pellets using a pressure of 8 tons for 1 min. The pellets were analyzed using a Fourier transform infrared (FTIR) spectrophotometer (Spectrum GX, PerkinElmer) which performed 16 scans over the range 370–4000 cm^{-1}. A scanning electron microscope (SEM) (JEOL model JSM-6301F) was used to observe the changes in size and morphology of the samples. An accelerating voltage of 15 kV was used to obtain the SEM images. The nanocrystals of particles were also examined by a transmission electron microscope (TEM) (JEOL model JEM-2010). For the mechanical testing, the loads for the compressive testing of the FSHA and sHA were dried cylindrical scaffolds of 7 mm in diameter and an average height of around 13 mm (height/diameter ≈ 2) were tested with a crosshead speed of 5 mm/min using a mechanical tester (Universal Testing Machine (Instron Model 55R4502, S/NH3342)).

2.5. In vitro biomineralization of sHA and FSHA scaffolds

To study the bioactive behaviors of sHA and FSHA scaffolds, each of the prepared powders was pressed in pellets (having diameters of ~1.3 cm and ~2.5 mm in thickness) and then soaked in a simulated body fluid (SBF) for 7 days. The SBF solution we used is one of the more extensively used ones (see ref. [21]). It contains the following chemicals: NaCl (136.8 mM), $NaHCO_3$ (4.2 mM), KCl (3.0 mM), K_2HPO_4 (1.0 mM), $MgCl_2 \cdot 6H_2O$ (1.5 mM), $CaCl_2$ (2.5 mM) and Na_2SO_4 (0.5 mM) and is buffered at pH 7.4 with tris(hydroxymethyl) aminomethane $[(CH_2OH)_3CNH_2]$ and hydrochloric acid (HCl). The bioactivity of each of the sHA and FSHA samples was assessed by immersing the pellets in 50 mL of SBF for one week at 37 °C. The SBF is replaced every three days to avoid any changes in the cationic concentration that may occur due to degradation of the sample. After immersion in the SBF, the pellet sample was washed with de-ionized water before the SEM analysis.

2.6. Attachment and morphology of cell on glass, FSHA and sHA substrates (biological test)

For the biological test, as previously described in Ref. [21], rat osteoblast-like UMR-106 cells (derived from Sprague–Dawley rats which are rapidly growing osteoblast-like cells. They usually become confluent within 3–4 days in 6-well plate or culture disc) [American Type Culture Collection (ATCC) No. CRL-1661] were grown in a Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria) and 100 U/mL penicillin–streptomycin (Gibco, Grand Island, NY, USA). The cells were propagated in a 75-cm^2 T-flask (Corning, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. The media also contained a pH indicator (phenol red), which becomes yellow in acidic pH and was sub-cultured as described in the ATCC protocol. Confluent UMR-106 cells used for the experiment were obtained by seeding cells at 1×10^6 cells/Petri dish into 100-mm petri dish in which circular glass (diameter ~1.3 cm), FSHA and sHA discs (diameter ~1.3 cm) had been placed. The Petri dishes were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was replaced every 3 days. At days 3, 5 and 7 after seeding, the glass, FSHA and sHA discs were examined for cell attachment and