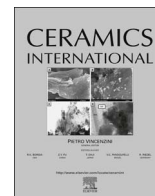




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# Natural origin hydroxyapatite scaffold as potential bone tissue engineering substitute

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## ABSTRACT

Fish scales derived natural hydroxyapatite (FS-HAp) scaffolds were prepared through solvent casting technique, which could mimic the structure of cortical and cancellous bone tissues of body system. The hydroxyapatite (HAp) biomaterial was synthesized by thermal decomposition of chemically treated fish scales. Fabricated scaffolds were characterized through morphological analysis, volumetric shrinkage, mechanical tests, and *in vitro*, *in vivo* biological studies. The projected scaffolds successfully mimic the cancellous/cortical bone system in terms of structure, porosity, mechanical strength, and exhibit excellent bioactive behavior. The FS-HAp scaffolds manifest good mechanical behaviors with Vickers Hardness (HV) of  $\sim 0.78$  GPa, 0.52 GPa compressive stress, 190 MPa tensile stress and  $\sim 35\%$  porosity on sintering at 1200 °C. *In vitro* and *in vivo* studies suggest these nontoxic HAp scaffolds graft with osteoconductive support, facilitating new cell growth on the developed scaffold surface. The graded grafts have a great potential for application as traumatized tissue augmentation substitute, and ideal for load-bearing bone applications.

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## 1. Introduction

Musculoskeletal systems are becoming a major health concern for ageing population, congenital musculoskeletal disorder patients and accidental injuries. In human body system, bone is the second mostly implanted tissue after blood [1,2]. The use of bone grafts to treat, replace, or augment skeletal injuries and fractured bones was established by a great number of bone grafting scaffolds available at present. However, the exact combination of mechanically stable, bioactive, low cost scaffold materials required for these applications is still under investigation. Available biomaterials are of high cost due to their expensive synthetic routes or precursor chemicals. Moreover, their bioactivity is a major concern due to rejections in body system. Although the research in scaffold materials for hard tissue replacement had a huge success at laboratory scale during the last two decades, it is still thriving to get success in medical applications.

The major components of human bones are collagen and a substituted HAp (a natural bio-ceramic found in teeth). In human skeletal system, HAp is the most important thermodynamically stable calcium phosphate inorganic component in physiological condition. Potential applications of HAp include drug delivery, chromatography, catalyst, adsorptive matrix, bone tissue

engineering [3]. The key advantages of synthetic HAp are its biocompatibility, deliberate biodegradability in physiological conditions with good osteoconductive and osteoinductive capabilities [4]. Taniguchi et al. demonstrated that sintered HAp exhibits excellent biocompatibility with soft tissues including skin, muscle and gums [5]. Synthetic HAp has also been widely used to repair hard tissues such as bone, bone augmentation, coating of implants or fillers for bone and teeth augmentation. The low mechanical strength of pure HAp ceramics is the only disadvantage for their application as load-bearing scaffold. Recent advances in science and technology of biomaterial synthesis and scaffold development have reignited the interest for HAp associated bone tissue engineering applications. This interdisciplinary engineering has concerned much attention as a new therapeutic means that may overcome the problems involved in the contemporary artificial prosthesis transplantation. Different synthetic routes such as wet chemical precipitation, sol-gel, hydrothermal synthesis, thermal decomposition, pyrolysis, sonochemical, mechanochemical, and biogenic sources have been applied [6,7] for HAp synthesis. Vats and coworkers performed clinical studies of different scaffold materials for tissue engineering applications [8]. Hollister et al. worked on the optimization of design and fabrication of scaffolds to mimic tissue properties for their successful biological applications [9]. Johnson et al. [10] studied and reviewed the compression, flexural and tensile properties of calcium phosphate (CaP) and CaP-polymer composites for applications in bone substitution

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and repair. Appleford et al. performed *in situ* studies of HAp scaffolds for bone tissue repair [11]. On the other hand, Tran et al. [12] demonstrated improved *in vitro* and *in vivo* osteoblast functions such as adhesion, proliferation, and synthesis of bone-related proteins and deposition of calcium containing minerals on nanostructured metals, ceramics, polymers, and composites.

In the present work, we adopted a new approach for developing strong, macroporous scaffolds from synthesized FS-HAp. Fish scales consist mainly of collagen proteins, connective tissue proteins and HAp crystals [13]. The process of extraction of HAp crystals are well documented in our previous article [14]. HAp powders were also synthesized by wet chemical precipitation, using calcium nitrate and diammonium hydrogen phosphate as precursor chemicals. The synthesized HAp powders were characterized by X-ray diffraction (XRD), Fourier transformed infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) techniques. Synthesized HAp powders were utilized to develop porous scaffolds through solvent casting. Porosity and mechanical properties of the fabricated HAp scaffolds were associated to their good biological activities. With ~35% maximum porosity, the sintered FS-HAp scaffolds possess compressive strength of ~0.52 GPa, hardness of ~0.78 GPa and ~190 MPa tensile strength, which are within the range of cancellous bones. A new graft composition is developed, that could be a promising macroporous scaffold material to support *in situ* traumatized bone tissue. Compared to conventional bone tissue engineering scaffold materials, the new apatite scaffolds facilitate implant fixation and tissue ingrowth in hard tissue replacement applications. The results obtained on mechanical properties, cell culturing and *in situ* bone growth over these scaffold materials indicate their possible use as future bone prosthetic implants.

## 2. Experimental procedure

### 2.1. Synthesis of hydroxyapatite from fish scale

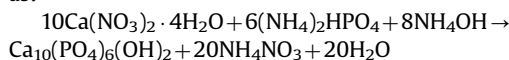
Tilapia fish (*Oreochromis mossambicus*) scales were collected and washed with tap water to remove attached undesired particles. To remove external hyaluronic acid and proteins, the initially washed scales were treated with 1 (N) NaOH (Sigma Aldrich) solution (2:1, v/w, NaOH Soln./fish scale) for 24 h at room temperature ( $25 \pm 3$  °C). The filtered fish scales are washed thoroughly with distilled water and dried at 50 °C in hot air oven for 6 h. Treated fish scales were calcined at 1200 °C for 2 h in air to obtain HAp bio-ceramics [15].

### 2.2. Synthesis of hydroxyapatite through chemical precipitation

Synthetic hydroxyapatite (SYN-HAp) powder was prepared by solution-precipitation method using calcium nitrate tetrahydrate [ $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (Sigma Aldrich)] and di ammonium hydrogen phosphate [ $(\text{NH}_4)_2\text{HPO}_4$  (Sigma Aldrich)] as starting materials and ammonia solution [ $(\text{NH}_4\text{OH}, 28\%)$  (Sigma Aldrich)] as precipitating agent [16].

A suspension of 0.24 M  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  [23.61 g  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  in 350 mL deionized water (18.2  $\Omega$  at 25 °C temperature)] was vigorously stirred at 40 °C. The pH of the solution was adjusted to 11.0 by adding drop-wise ammonia solution. Next another solution of 0.29 M  $(\text{NH}_4)_2\text{HPO}_4$  [(7.92 g  $(\text{NH}_4)_2\text{HPO}_4$  in 250 mL deionized water (18.2  $\Omega$  at 25 °C temperature)] was prepared. At 40 °C temperature freshly prepared 0.29 mL diammonium hydrogen phosphate solution was added drop-wise to the  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  solution. The transparent calcium nitrate solution became milky white in colour as the formation of HAp nanoparticles in the solution. The following reaction could be expressed

as:



The white precipitate was removed from the reaction solution by centrifugation at 5000 rpm for 6 min and dried at 80 °C. Obtained SYN-HAp powder was calcined in air at 1200 °C for 2 h.

### 2.3. Scaffold development

Solvent casting technique was employed to prepare porous HAp scaffolds (Fig. 1). First of all, 50 mL of 2% aqueous starch solution was prepared and synthesized HAp powders (either FS-HAp for FS-HAp scaffold or SYN-HAp for SYN-HAp scaffold) were added in steps to attain the desirable solids with 55 vol% loading. Insoluble starch (1 g) was added as porogen in this mixture. Next monomer, methacrylamide (MAM) 2.5 mL and cross-linker methylene bisacrylamide (MBAM) 0.5 mL was added. To initiate the chemical reactions 1  $\mu\text{L/g}$  of slurries of ammonium persulphate (10% APS) was used. The total mixture of solutions and powders were milled in rotary milling machines for six (6) hours at 300 rpm speed. Finally, 0.5  $\mu\text{L/g}$  of slurries and tetramethyl ethylenediamine (TEMED) were added to the mixture, which served as catalyst [17]. During mixing, some bubbles were formed in the slurry, and to avoid such uncontrolled bubble formation, octanol was used as de-foaming agent. The prepared slurry was finally casted into the desired mould and dried at controlled humidity at 50 °C for 48 h. After complete drying, the green bodies were removed from the mould and after measuring their sizes (diameter, width and height); they were sintered at 1200 °C temperature for 2 h in air atmosphere. After sintering, these scaffolds were tested to determine their shrinkage percentage. The same technique was employed for chemically synthesized HAp powder to prepare porous scaffolds.

## 3. Characterization

### 3.1. Characterization of HAp Powders

The calcined HAp powders were characterized by X-ray diffraction (XRD) using Cu K $\alpha$  radiation ( $\lambda = 1.5405$  Å) of a Bruker D8 Discover diffractometer, operating at 30 keV and 25 mA. FTIR analysis (PerkinElmer Spotlight 400 FT-IR) of the samples was performed in the 4000–400  $\text{cm}^{-1}$  range to determine the functional groups present on their surface. Field-emission scanning electron microscopy (FE-SEM, SUPRA 40, CARLZEISSMT, Oxford) and energy dispersive spectroscopy (EDS) analysis were performed to determine the morphology and composition of the samples, as well as to observe the pores developed in the scaffolds.

### 3.2. Mechanical characterizations of scaffold

#### 3.2.1. Hardness

The Vickers test, a square-based pyramidal diamond indent was used, whose opposite sides met at the apex at an angle of 136° [18]. The diamond indent was pressed over the surface of the material at loads ranging up to approximately 0.5 Kgf. The size of the impression (maximum 0.5 mm) was measured with the aid of Wilson Vickers hardness testing machine.

The Vickers Hardness (HV) was calculated using the formula:

$$HV = \frac{F}{A} = \frac{1.8544F}{d^2}$$

where A is the surface area of resulting indentation in  $\mu\text{m}^2$ , d = the average length of the diagonal left by the indenter in  $\mu\text{m}$ , F = applied force in Kgf. Fully polished HAp samples with smooth

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