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Chemical Synthesis, Characterization and Bioactivity Evaluation of Hydroxyapatite Prepared from Garden snail (*Helix aspersa*)

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Abstract

A novel attempt is made to convert the calcium carbonate skeleton of widely available garden snail shell (*Helix aspersa*) to hydroxyapatite based bioceramics. The snail shell was found to decompose within 850^{0}C to all the carbonate phases. The calcined snail shells were then treated with acids followed by different chemicals in ammoniacal media maintaining proper stoichemetry to produce fine Hydroxyapatite (HAP) as filter cake with Ca/P molar ratio of 1.67. The dried HAP powder was extremely pure with specific surface area of 15m^2/g. The different characterization techniques were adopted both for calcined snail shell and HAP synthesized like X-ray Diffraction (XRD), Thermal Analysis (DTA/TGA), Fourier Transform Infra red Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). The surface area and the particle size, of the HAP powder prepared by chemical precipitation route, were also determined by BET and Malvern particle size analyzer respectively. The synthesized powder was soaked in stimulated body fluid (SBF) medium for various periods of time in order to evaluate its bioactivity. The changes of the pH of SBF medium were measured. High bioactivity of prepared HAP powder due to the formation of apatite on its surface was observed.

Keywords: Snail shell; Hydroxyapatite; Calcination; β-tricalcium phosphate; Bioactivity

Introduction

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] based bioceramics are successfully used as implants as it is chemically similar with the inorganic constituent of biological hard tissue [1]. It is present in bone, teeth and tendons to give these organs stability, hardness and function. On account of its chemical similarity with the biological calcified tissue it is remarkably biocompatible [2]. Due to the formation of strong bond with the hard tissue, it is widely used in orthopedics or in dental implants.

HAP is also a potential implant material due to its excellent osteoconductive properties [3,4]. HAP has been shown to stimulate osteoconduction and is a material that can be integrated into bone without provoking an immune reaction. The biological response to HAP implants is influenced by its properties. The application of HAP as useful biocompatible materials largely depends on the purity and morphology of the powder. HAP can be prepared by different routes like chemical precipitation, sol-gel route, combustion synthesis, plasma etc [5-10]. The purity in the final HAP powder and stoichemetry (molar ratio of Ca/P = 1.67) can be well controlled in chemical precipitation route. The different chemical processes use precursors like $Ca(NO_3)_2$, $Ca(OH)_2$ etc. as the source of Calcium[Ca] and $(NH_4)_2HPO_4$, H_3PO_4 etc. as the source of Phosphorus [P] during synthesis of HAP. The extremely pure HAP powder is very costly and needs high quality precursors. The most of the sources of Ca^{2+} contains different types and level of impurities mainly silica. Snail shell consists of $CaCO_3$ with minor amount of $MgCO_3$ and other matters, can be potential precursors for the production of HAP.

The composition of human bone is an inorganic/organic hybrid consisting of 70% (wt) apatitic calcium phosphates and 30% (wt) organic (largely collagen) [11]. The apatitic calcium phosphate of bone mineral consists of carbonate, small amount of sodium, magnesium and other trace elements. The submicroscopic crystals of calcium phosphates in bone resemble the crystal structure of synthetic HAP[12].

The objective of the present paper is to synthesize pure and biocompatible HAP using snail shell as precursor following chemical precipitation method [13]. The powders are characterized using XRD, DTA/TGA, FTIR, SEM and BET surface area. Hydroxyapatite prepared by precipitation route also has the feature of small size, low crystallinity and high surfacial activation which can meet different demands [14].

Keeping the above points in mind, the present study was aimed to produce and to enhance the bioactivity of stoichemetric HAP prepared from garden snail shell (*Helix aspersa*) and to evaluate its bioactivity in simulated body fluid.

Materials and Methods

Garden snail shells (SS) were collected and their shell covering was removed carefully. Shells were washed with tap water followed by distilled water to remove the mud, sand and other impurities. The cleaned shells were dried in the direct sunlight for 2 days. Dry and cleaned SS were calcined at 1000^{0}C for 2 hours so that all organic matters and proteins escape out. The calcined SS was treated with concentrated nitric acid to convert it to $Ca(NO_3)_2$. 130 ml. of 1.63(N) ammonical $Ca(NO_3)_2$ solution was added drop wise to a mixture of ammonical $(NH_4)_2HPO_4$ solution with constant stirring with the help of magnetic stirrer. The pH of the solution was maintained at 10. Hydroxyapatite was formed as per the following reaction:

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 2H_2O \longrightarrow Ca10(PO_4)_6(OH)_2 + 12NH_4NO_3 + 8HNO_3$$

The resulting suspension was boiled for 10 minutes and cooled in an ice bath overnight to obtain a white gelatinous precipitate. The pre-

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cipitate was filtered and filtered cake (residue) was dried in the oven at 800C. The dried sample of hydroxyapatite was ground to powder.

The thermal analysis of the Snail shell was performed by (NETZSCH-Geratebau GmbH Thermal Analyser) at a heating rate of 10^0C/minutes from ambient to 1200^0C to study the weight loss and thermal behavior. The powder samples of SS, calcined SS and synthesized HAP samples were examined with high resolution X-ray Diffractometer (PW-1830, Philips, Netherlands) using Cu-Kα radiation. The X-ray diffraction (XRD) patterns were recorded in the steps of 0.01^0 interval with 1s counting time at each step. Fourier transform infra red (FTIR) spectrum of synthesized HAP powder was obtained over the wave numbers 400 – 4500cm^{-1}. The powder was dispersed into pellets of KBr (mixed in 1:4 ratio) and the spectra was recorded with a Perkin-Elmer (S2000) IR spectrometer. The particle size analysis of HAP was done by Malvern Particle Size Analyzer (Model – Micro-P, UK). The surface morphology of SS and the prepared HAP powder were studied by Scanning Electron Microscope (SEM) while the surface area of the prepared HAP samples was determined with BET surface area analyzer (QUANTACHROME Model:Autosorb1).

In vitro bioactivity evaluation

The *in vitro* bioactivity evaluation of synthesized HAP powder from garden snail shell was performed in a stimulated body fluid (SBF) media of pH 7.4 at a ratio of 1 mg/ml in a water bath at 37°C. The changes in the pH of SBF medium were measured at pre-determined time intervals using a pH meter. Scanning electron microscopy (SEM) was used to identify the apatite formation on surface of the samples and to evaluate the surface morphology of the samples after immersion in SBF medium for 2, 8 and 15 days respectively.

Preparation of (Synthetic Body Fluid) SBF

SBF is known to be a metastable buffer solutions [15,16] and even a small, undesired variance in both of the preparation steps and the storage temperatures, may drastically affect the phase purity and high-temperature stability of the produced HA powders, as well as the kinetics of the precipitation processes.

Merck-grade NaCl (99.5%), $NaHCO_3$ (99.5%), KCl (99.0%), $Na_2HPO_4).2H_2O$ (99.5%), $MgCl_2).6H_2O$(99.0%), Na_2SO_4, $(CH_2OH)_3CNH_2$ (99.5%), $CaCl_2.H_2O$(99.0%) and HCl (37 vol%, Carlo-Erba, Rome, Italy) were used in the preparation of the SBF of this study.

SBF solutions [17-21] were prepared by dissolving appropriate quantities of the above chemicals in deionized water. Reagents were added, one by one after each reagent was completely dissolved in 700 ml of water, in the order given in Table 1. A total of 40 ml of 1 M HCl solution was consumed for pH adjustments during the preparation of 1 l of SBF solutions. A 15 ml aliquot of this acid solution was added just before the addition of the sixth reagent, viz., $(CaCl_2)$ $2H_2O$. Otherwise, the solution would display slight turbidity. The remaining part of the HCl solution was used during subsequent titration. Following the addition of the eight reagent (*tris*(hydroxymethyl) aminomethane), the solution temperature was raised from ambient to 37^0C. This solution was then titrated with 1 M HCl to a pH of 7.4 at 37^0C. During the titration process, the solution was also continuously diluted with consecutive additions of de-ionized water to make the final volume equal to 1 l. It was observed in this study that the prepared SBF solutions can be stored at 5^0C for a month without degradation.

Biodegradation Test

Biodegradation test of calcined HAP prepared from garden snail shell (*Helix aspersa*) was done by taking Tris-HCl buffer solution. 0.05MTris- HCl solution was prepared using distilled water. The pH of solution was maintained 7.4 at 37^0c by adding 1MHCl. Calcined HAP in the form of pallets were soaked in Tris-HCl buffer solution for one week then the samples were dried at 100^0c and final weight loss of sample was determined by the formulae as given below:.

$$\% \text{ Weight Loss} = \frac{W_1 - W_2 \times 100}{W_2}$$

where, W_1 = initial weight of sample

W_2 = final weight of sample after soaking in Tris-HCl solution.

Results and Discussion

Dta/tga of snail shell (ss)

Figure 1 Showed the DTA/TGA analysis of Snail Shell showed the weight loss at temperature between 90^0C – 120^0C that is due to the physically adsorbed water. Over a wide range of temperature from 250^0C – 400^0C the weight loss is due to the decomposition of $MgCO_3$ combined with the combustion of hydrocarbons. The weight loss along with endothermic peak at 750^0C - 850^0C indicates the decomposition of $CaCo_3$ following the reaction.

$$CaCO_3 \longrightarrow CaO + CO_2 \uparrow$$

So it is confirmed from the thermal analysis that Snail Shell mainly contains $CaCO_3$ along with small amount of $MgCO_3$ and other organic matters.

XRD analysis

A typical XRD profile of SS and calcined SS HAP has been shown in Figure 2. The raw SS showed the presence of $CaCO_3$ phase, where as CaO was detected in the calcined Snail shell. The appearance of calcined SS was soft, porous and white in colour. However, due to delay in recording some amount of CaO was converted to $Ca(OH)_2$ by adsorbing moisture from the atmosphere which is depicted in Figure 2b.

Order	Reagent	Amount (gpl)
1	NaCl	6.547
2	NaHCO	2.268
3	KCl	0.373
4	$Na_2HPO_4.2H_2O$	0.178
5	$MgCl_2.6H_2O$	0.305
6	$CaCl_2.2H_2O$	0.368
7	Na_2SO_4	0.071
8	$(CH_2OH)_3CNH_2$	6.057

Patent pending. "Turkish Patent Institute," Turkey, *Appl*. No. 99-0037, 11 January (1999)

Table 1: Chemical composition of SBF solutions.

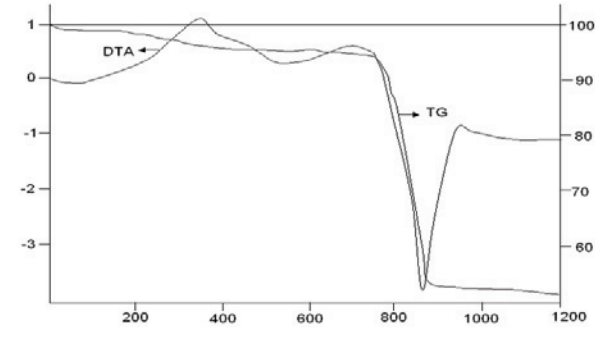

Figure1: Shows the TGA/DTA analysis of as dried Snail shell.

The XRD phase analysis of HAP powder has been shown in Figure 3. Three-high-intensity peaks located at 2θ = 31.7°, 32.2° and 32.9° with Cu-Kα radiation are difficult to be exactly recognized from their diffraction patterns. XRD patterns reveals the formation of HAP and is well resembled with the standard JCPDS file. The unindexed peak at 30.75° Figure 3b may be due to β-tricalcium phosphate; which indicates the initiation of conversion of HAP to β-tricalcium phosphate on heating HAP above 800°C. The calcined HAP exhibits well crystallized sharp peaks of characteristics HAP. The HAP powders, thus synthesized from Snail Shell precursor, are very pure and chemical analysis of powders confirms the same observation.

FTIR analysis

Infrared characterization was carried out for the sample to study the spectral characteristics indicative of the chemical bonding in the synthesized HAP powder. The spectrum Figure 4 can be divided into four regions with peaks having wave numbers around 3500, 1420, 1100 and 600cm^{-1}. The peak observed around 3431.8cm^{-1} is due to the presence of –OH bond [22]. This peak is mainly due to O-H stretching vibration in HAP [23]. The peak at 1036.2 cm^{-1} is associated with the stretching modes of the P-O bonds of HAP [23,24]. The double peak at 603.1 cm^{-1} and 567.4cm^{-1} are due to bending modes of P-O bonds in phosphate groups [24]. Thus, the presence of PO_4^{3-} - group in HAP is almost confirm from IR studies. The pH of the medium during synthesis of HAP was maintained using ammonium solution and it was removed from the suspension with repeated washing with distilled water. In spite of all efforts to remove ammonia from the solution, there is a possibility of small amount of it in the HAP powder. The IR analysis shows a small broad peak at 1422.6cm^{-1}; which is characteristics peak of NH_4^+- group [25-27].

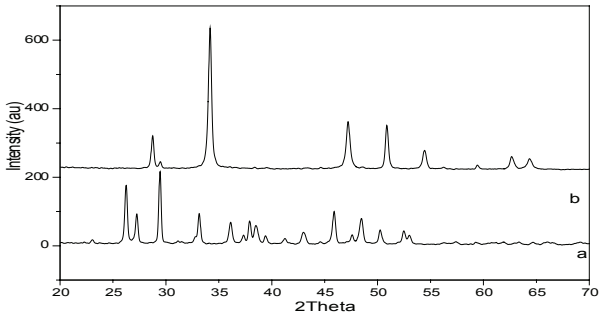

Figure 2: XRD patterns of (a), Snail shell; (b), Calcined snail shell at 1000°C/2Hr.

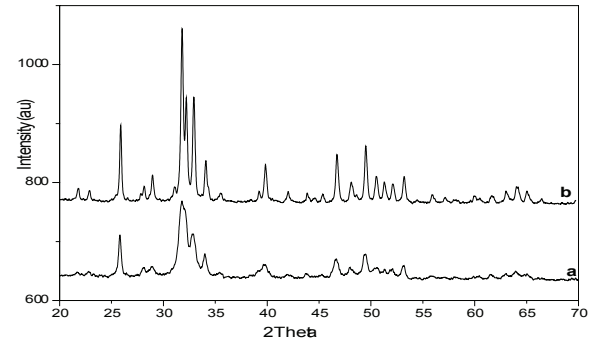

Figure 3: XRD Pattern of: (a) HAP(synthesized), (b) Calcined HAP (synthesized) 800°C/2Hr.

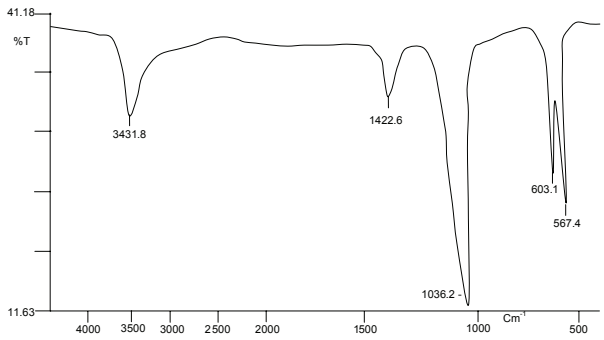

Figure 4: FTIR Spectrum of HAP powder.

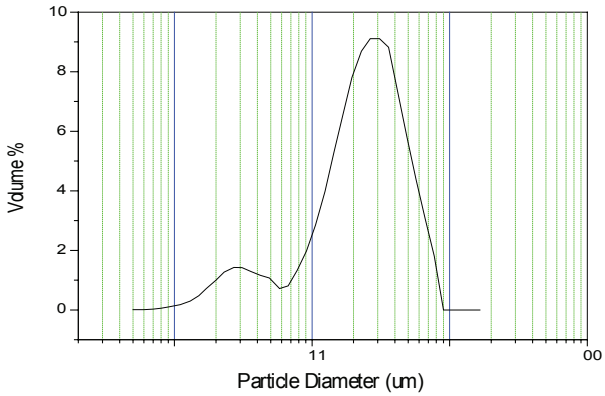

Figure 5: Particle Size Analysis of HAP powder.

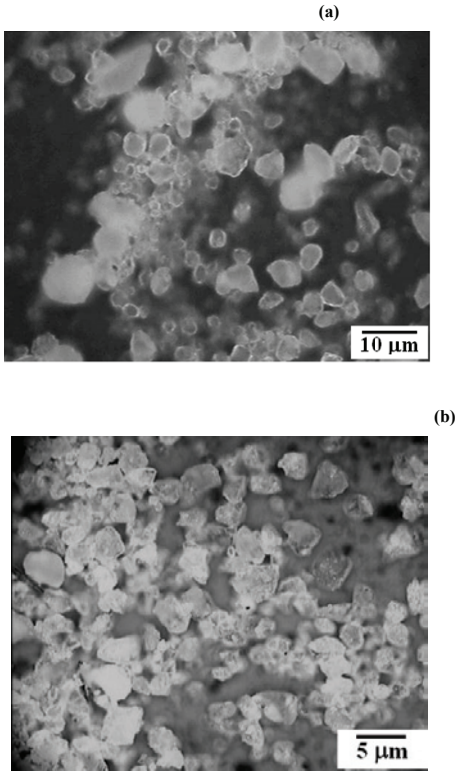

Figure 6: SEM Micrograph of (a) Synthesized HAP powder (b) Calcined (850°C/2h) HAP powder.

Particle size analysis

Particle size analysis of HAP powder was carried out following Laser technique and pattern of particle size distribution is plotted in Figure 5. Average particle size was found to be 2.63 μm. Small amount of fine particles (0.2-0.3 μm) are also present in the synthesized powder.

Surface area measurement

The surface areas of the hydroxyapatite powder and calcined HAP are determined are 83 and 15m^2/gm respectively. Powders are agglomerated during calcinations; but HAP powders have to be calcined to remove volatile impurities like ammonia.

Scanning electron microscope (SEM)

The morphologies of as synthesized and calcined HAP powders are shown in Figure 6. Uncalcined HAP powders are almost regular and round in shape; where as calcined HAP powders are agglomerated. The microstructure as reveals from SEM is in well- agreement with the particle size analysis and BET surface area analyzer results.

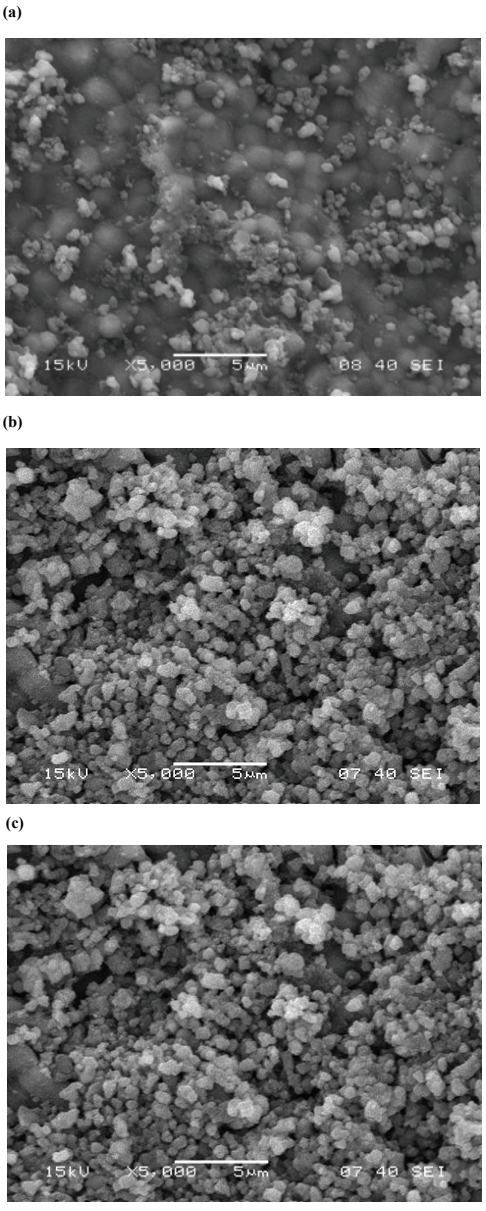

Figure 7: SEM micrographs of (a) the surfaces of synthesized HAP powders after soaking in SBF for 2 Days (b) the surfaces of synthesized HAP powders after soaking in SBF for 8 Days (c) the surfaces of synthesized HAP powders after soaking in SBF for 15 Days.

Bioactivity evaluation

The SEM micrographs of the surfaces of the immersed HAP powder after soaking in SBF for various periods of time are shown in Figure 7. Tiny agglomerated bone-like apatite particles could be formed on the surface of the HAP powders.

Figure 7 shows that tiny agglomerated bone-like apatite particles could be formed on the surface of the HA powders soaked for 2 days, 8 days and 15 days respectively. The number and the size of these agglomerated particles increased with increasing soaking times. Identification and evaluation of apatite formation on the surface of a material in SBF is useful for predicting the *in vivo* bone bioactivity of the material, not only qualitatively but also quantitatively [28-31]. The results indicated that the synthesized HAP powder from Garden Snail shell (*Helix aspersa*) showed the high bioactivity in SBF solution.

In-vitro biodegradation

Biodegradation of calcined HAP samples in the form of pallets were carried out in Tris-HCl solution. HAP samples were soaked in Tris-buffer solution at pH 7.4 and temperature 37°C for 7 days [32]. When porous HA was soaked in Tris-buffer solution, the loss of calcium ion took place which resulted in the increase in pH of the buffer from 7.4 to 8.2 which confirms the biodegradation of HAP. The calcined HAP prepared from garden snail shell by chemical precipitation method showed weight loss of 4.5%. Thus it appears that the ageing time in Tris-HCl solution may also affect the weight loss behavior.

Conclusions

A stoichiometric, pure and thermally stable hydroxyapatite powder was synthesized from Snail shell (*Helix aspersa*) by chemical precipitation method. XRD analysis indicated the phase purity and crystallinity of hydroxyapatite powder. TG/DTA result showed that Snail shell is mainly composed of calcium carbonate ($CaCO_3$). Fine particle size of hydroxyapatite was produced. The present work is based on the utilization of biological waste (Snail shell) to produce hydroxyapatite for Bio-medical applications. The prepared HAP powder showed high bioactivity similar to that in biological apatite and higher bioactivity in comparison with conventional HAP. Thus, prepared HAP from garden snail shell (*Helix aspersa*) might be more useful for treatment of oral bone defects in comparison with conventional HAP, and might be more effective as a bone replacement material to promote bone formation.

An attempt will be made in future to synthesize porous HAP and study its bio-compatibility. Mass production of biocompatible HAP for biological application may be possible at simple and low cost through this route.

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Competing Interests

The authors have no competing interests with the work presented in this manuscript.

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