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## Morphological Diversity in Nanohydroxyapatite Synthesized from Waste Egg Shell : Verification and Optimization of Various Synthesis Parameters

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### **Abstract:**

*Biomimetic rod shaped hydroxyapatite (HA) was prepared from calcined eggshell waste with a particle width of 16 nm. The method adopted mainly aimed at preparing nanosized deagglomerated biocompatible HA by eco-friendly synthesis route. The method of preparation used sucrose as a dispersing medium to enable size reduction, morphology control and ready removal of dispersing agent at a temperature around 550 °C. Reported methods in existing literature mainly used organic templates as morphology regulators, the traces of which if left unremoved upon calcination can pose toxicological issues as far as their application in bio- realms are concerned. Spherical shaped HA of 19 nm particle diameter resulted when the calcium precursor used was uncalcined egg shell. The role of sucrose was confirmed by carrying out a control sample preparation in water, the powders obtained showed presence of agglomeration with an increased particle size. X-ray diffraction analysis (XRD) and Fourier transform infrared spectroscopy (FTIR) studies confirmed the formation of pure HA . Scanning Electron Microscope (SEM) photographs gave the morphological diversities of different HA samples. Transmission Electron Microscopy (TEM) images substantiated the formation of uniform rod shaped HA. Elemental composition was confirmed by inductively coupled plasma– atomic emission spectroscopy (ICP-AES) and energy dispersive X-ray spectroscopy (EDXS)*

**Keywords:** Nano hydroxyapatite ; wet chemical precipitation; morphology; crystallinity; calcined egg shell; uncalcined egg shell

### **1. Introduction**

Hydroxyapatite (HA) with the molecular formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  is the main mineral component of hard tissue. Synthetic hydroxyapatite is used in biomedical and dental applications due to its similarity to main mineral component of the hard tissue of human body such as bone, dental enamel etc. and also its excellent biocompatibility, bioactivity and osteoconductivity [1,2]. There are several reported methods for preparing hydroxyapatite such as chemical precipitation [3-6], sol-gel process [7, 8], hydrothermal method [9,10], biomimetic deposition [11]. However, among these methods wet chemical method is more promising owing to its ease of production, low working temperature, inexpensive equipment etc and also high percentage of purity [12]. As distinct from microcrystalline HA, nanosized powders have high specific surface area and therefore exhibit enhanced activity towards chemical and biological interactions in the human body [13, 14, 15]. The size, crystallinity, morphology of the synthesized particles depend on the method of preparation, raw materials used, concentration of starting materials, pH of the system, stirring speed, temperature, aging time etc

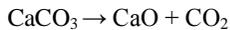
The main component of synthetic hydroxyapatite (HA) is calcium. Calcium needed for the preparation of HA from living bodies reduces the chance of impurities and reduce the cost of production [16, 17]. Various natural sources like bovine bone, corals, fish bone, egg shells have been recognized so far due to their advantage of biological origin as well as a method for recycling of inorganic waste [18,19,20]. The eggshells constitute 11% of the total weight of the egg and are composed of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%) [21]. According to the Food and Agricultural Organization of the United Nations, the world's egg production was approximately 64 million metric tons in 2010, and India occupies the third position with 3.4 million metric tons. A huge amount of egg shells is being produced daily which are basically of no use causing environmental pollution [22]. So utilization of shell will benefit in two ways, egg shell derived HA will be cost effective material and utilization of egg shell reduces environmental pollution and related problems.

Many researchers have already reported the preparation of HA using eggshell as a calcium source [19- 23]. Although various works have been done focusing size reduction of HA powders, the morphological differences among the various eggshell derived HA samples were the least discussed. The present work emphasizes the effect of calcium precursor, medium and temperature on morphology along with size and crystallinity of the HA samples. For this the synthesis was conducted using calcined and uncalcined egg shell in water as well as sucrose medium at different heating temperatures.

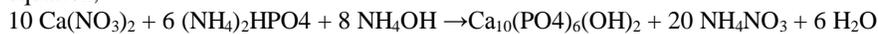
## 2. Experimental

### 2.1. Material Synthesis

Waste egg shells collected from local catering unit was cleaned well and boiled in water for 30 minutes. It was then dried in microwave oven, crushed and powdered. The powdered egg shell was calcined in a muffle furnace at a temperature of 900 °C for 4 hours to convert it into calcium oxide according to the equation,



The calcium oxide thus obtained was converted to calcium nitrate by dissolving in requisite amount of con.HNO<sub>3</sub>. The solution was made up to a definite volume and the amount of calcium was determined by EDTA method. 100 ml of this solution (~1M) was added to 1 litre 0.1 molar sucrose solution with stirring. Ammonia solution (1:1) was added dropwise to keep the pH 10-11 and the mix was heated to 70 °C. To this hot solution stoichiometric amount (Ca:P = 10:6) of 0.5 molar diammonium hydrogen phosphate solution was added in drops with vigorous stirring. White gelatinous precipitate of hydroxyapatite was formed. Ca(NO<sub>3</sub>)<sub>2</sub> was reacted with di-ammonium hydrogen phosphate in ammoniacal medium to form hydroxyapatite according to the equation,



The solution was stirred for another one hour and left for aging for 24 hours. The precipitate was filtered, washed and kept for vacuum drying at 65 °C for 36 hours. It was then heated in a muffle furnace at 550 °C and 900 °C each for 4 hours and are referred to in this paper as H<sub>1</sub>Su-550 and H<sub>1</sub>Su-900 respectively. The same procedure was repeated using uncalcined egg shell and designated in this paper as H<sub>2</sub>Su-550. The sample H<sub>1</sub>W-550 was prepared from calcined eggshell in water medium through the

Sample name	Calcium precursor	Medium of reaction	Final heating Temperature
H <sub>1</sub> Su-550	Calcined Egg shell	0.1M sucrose solution	550 °C
H <sub>1</sub> Su-900	Calcined Egg shell	0.1M sucrose solution	900 °C
H <sub>2</sub> Su-550	Uncalcined Egg shell	0.1M sucrose solution	550 °C
H <sub>1</sub> W-550	Calcined Egg shell	Water	550 °C

same route.

Table 1: Preparation parameters of HA

### 2.2. Characterization of Hydroxyapatite

#### 2.2.1 Fourier transform infra-red spectroscopy

The chemical nature and molecular bond structure of the samples were confirmed using Fourier transformed infra-red spectroscopy (FTIR). FTIR transmittance of the samples in the 4000-400 cm<sup>-1</sup> region were recorded on a Thermo Nicolet FTIR Spectrometer Model Avatar 370 by using KBr pellet technique.

#### 2.2.2 X-ray Diffraction Analysis

The crystallographic phases of the HA samples were identified by using X-ray diffractograms (XRD) (Bruker D8 advanced model employing Cu K $\alpha$  radiation  $\lambda=1.54 \text{ \AA}$ ). The XRD patterns were recorded in the 2 $\theta$  range of 0-70° with a step size of 0.02° using a tube voltage of 40 kV and tube current of 35 mA.

The crystalline size of the sample was determined by Debye- Scherrer formula

$$D = 0.9 \lambda / \beta \cos \theta$$

where D represents the mean grain size,  $\beta$  represents full width at half maximum (FWHM),  $\lambda$  is the diffraction wavelength (0.154 nm) and  $\theta$  is the diffraction angle.

#### 2.2.3 Scanning Electron Microscopy

The morphological characterization of HA samples was carried out using (JEOL JSM 6390 L V) Scanning Electron Microscope (SEM). The samples were subjected to gold sputtering prior to electron microscopy to give the necessary conductivity.

#### 2.2.4 Transmission Electron Microscopy

A high resolution transmission electron microscopy (HR-TEM), FEI model TECNAI G2 F 30 instrument operated at an accelerating voltage of 300 KV (Cs = 0.6mm, resolution 1.7 $\text{\AA}$ ) was used for analyzing the particle size and surface morphology of the sample. For TEM studies suspensions of HA sample in distilled water were prepared and a drop of this suspension was placed on a 200 mesh copper grid coated with carbon film (ICON Analytical). This was subsequently dried under vacuum overnight and then loaded in electron microscope chamber.

### 2.2.5 Chemical Analysis

The chemical composition of synthesized apatite was analyzed using an inductively coupled plasma– atomic emission spectroscopy (ICP-AES) in Thermo Electron IRIS INTREPID II X SP DUO spectrometer. The sample for the analysis was prepared by first drying the HA powder in an oven at 120°C for 1 hour and then dissolving approximately 100 mg of dried sample in 7 ml of reagent grade concentrated nitric acid. The solution was later made up to 100ml using distilled water so that the concentration of various elements was within the linear range of detection for the ICPAE spectrometer. The solution was analyzed for concentration of calcium, phosphorous, magnesium, sodium and strontium. The elemental composition of the sample was also analyzed using energy dispersive X-ray spectroscopy (EDXS) coupled with SEM.

## 3. Results and Discussion

### 3.1. FTIR analysis

Figure 1 (a), (b), (c) and (d) show FTIR spectra of HA samples, H<sub>1</sub>Su-550, H<sub>1</sub>Su-900, H<sub>2</sub>Su-550 and H<sub>1</sub>W-550 respectively. The infrared band positions and their assignments are given in Table 2.

Assignments	Vibrational frequencies (cm <sup>-1</sup> )				
	Standard.HA	H <sub>1</sub> Su-550	H <sub>1</sub> Su-900	H <sub>2</sub> Su-550	H <sub>1</sub> W-550
Structural OH <sup>-</sup>	3572	3571	3571	3572	3571
H <sub>2</sub> O adsorbed	3420	3431	3431	3431	3431
H <sub>2</sub> O adsorbed( $\nu_2$ )	1630	1633	-	1634	1633
PO <sub>4</sub> <sup>3-</sup> bending ( $\nu_3$ )	1094	1094	1094	1093	1093
PO <sub>4</sub> <sup>3-</sup> bending ( $\nu_3$ )	1040	1038	1040	1040	1036
PO <sub>4</sub> <sup>3-</sup> stretching( $\nu_1$ )	962	961	961	960	961
Structural OH <sup>-</sup>	631	632	632	633	632
PO <sub>4</sub> <sup>3-</sup> bending ( $\nu_4$ )	601	602	602	602	602
PO <sub>4</sub> <sup>3-</sup> bending ( $\nu_4$ )	574	568	568	568	564
PO <sub>4</sub> <sup>3-</sup> bending ( $\nu_2$ )	472	472	471	472	473

Table 2: Assignments of vibrational frequencies of different HA samples

Absorption peaks corresponding to (PO<sub>4</sub>)<sup>3-</sup> and OH<sup>-</sup> groups which are characteristics of hydroxyapatite [24] are visible in the spectrum of all the four samples. The peaks at 3571-3572 and 631-633 cm<sup>-1</sup> are assigned to the stretching vibrations of the hydroxyl group in crystal structure of hydroxyapatite which may be originating from libration bands of OH<sup>-</sup> group [25]. The peaks at 3431 cm<sup>-1</sup> and 1631-1633 cm<sup>-1</sup> correspond to the bending modes of hydroxyl group in the adsorbed water. At the same time these peaks are absent or with very low intensity in the sample prepared at 900°C (H<sub>1</sub>Su-900), which shows the removal of the adsorbed water at higher temperature. Peaks at 1093- 1094 cm<sup>-1</sup> ( $\nu_3$ ), 1037-1040 cm<sup>-1</sup> ( $\nu_3$ ), 960-962 cm<sup>-1</sup> ( $\nu_1$ ) and 564- 568 cm<sup>-1</sup> ( $\nu_4$ ), 471-472 cm<sup>-1</sup> ( $\nu_2$ ) indicate the presence of (PO<sub>4</sub>)<sup>3-</sup> groups in the hydroxyapatite.

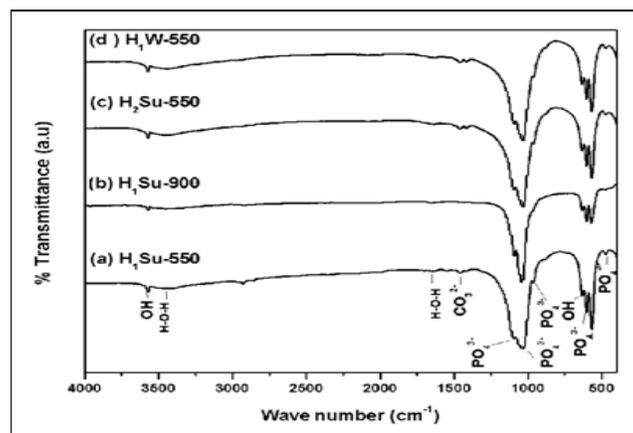


Figure 1: FTIR of of HA prepared from (a) calcined egg shell in sucrose medium at a final heating temperature of 550°C H<sub>1</sub>Su-550, (b) calcined egg shell in sucrose medium at a final heating temperature of 900°C H<sub>1</sub>Su-900 (c) uncalcined egg shell in sucrose medium H<sub>2</sub>Su-550 (d) calcined egg shell in water H<sub>1</sub>W-550

The presence of low intensity peaks in the region 1400 cm<sup>-1</sup> - 1460 cm<sup>-1</sup> is due to the presence of carbonate ions. The incorporation of carbonate ions in the apatite structure is believed to have taken place during the preparation of HA, where the atmospheric CO<sub>2</sub> reacts with OH<sup>-</sup> in the HA, forming carbonate ions [26]. Since carbonates are constituents of bone structures [27] the presence of CO<sub>3</sub><sup>2-</sup> may improve the bioactivity of HA rather than being blamed as an impurity. But the spectrum of the

sample heated at 900 °C is completely devoid of this carbonate peak which confirms the elimination of carbonate group at higher temperature. There is no marked evidence for any other impurities.

### 3.2. XRD Studies

XRD patterns of HA samples, H<sub>1</sub>Su-550, H<sub>1</sub>Su-900, H<sub>2</sub>Su-550 and H<sub>1</sub>W-550 are shown in Figure 2 (a), (b), (c) and (d) respectively. The intense peaks appear in the 2θ range of 20 – 60, characteristic of the hexagonal apatite phase (JCPDS 09-432). Characteristic peaks, for instance at (002), (211), (300), (202), planes were observed. A high intensity peak from (211) plane confirms the formation of hydroxyapatite. The absence of any other distinguished peak confirms the formation of pure monophasic hydroxyapatite. The peaks corresponding to sample H<sub>1</sub>W-550 which was prepared from calcined egg shell in water medium in figure (d) is somewhat deviating from the baseline and is an indication of more amorphous nature compared to other samples which were prepared in sucrose medium. This shows that the change of medium from water to sucrose increases the crystallinity of the sample. The straight base line and the sharp peaks of the diffractogram in H<sub>2</sub>Su-900 confirmed the highly crystalline nature of the sample at higher temperature (28).

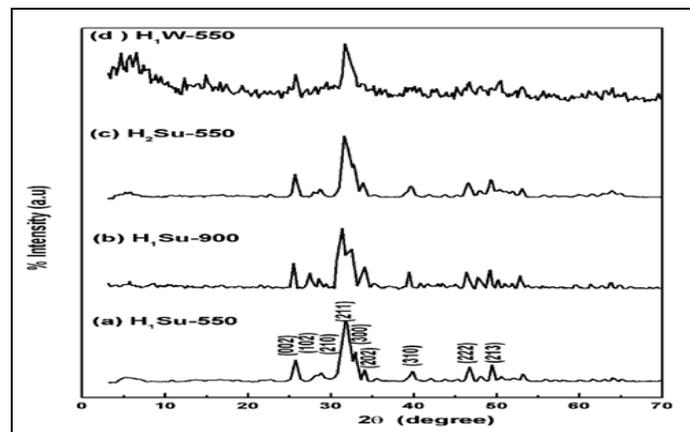


Figure 2: XRD pattern of (a) H<sub>1</sub>Su-550, (b) H<sub>1</sub>Su-900 (c) H<sub>2</sub>Su-550 (d) H<sub>1</sub>W-550

The broadening of the (002) peak was used to calculate an average grain size using Scherrer's equation. The samples were found to have grain sizes 16 nm, 19 nm, 27 nm, and 31 nm respectively for H<sub>1</sub>Su-550, H<sub>2</sub>Su-550, H<sub>1</sub>W-550, and H<sub>1</sub>Su-900. So it is evident that for the samples prepared at the same final heating temperature H<sub>1</sub>Su-550 is having the minimum size. The reduced size for the HA prepared in sucrose medium is due to the predominant adsorption of sucrose molecules onto the Ca-rich surface during the initial stages of crystallization which inhibits the growth of the particles. The nanoparticles may be formed by an oriented aggregation of primary crystallite domains along the c-axis direction. [29, 30].

### 3.3. SEM Analysis

The morphology of the HA samples are given in figures 3(a), (b), (c) and (d). It is obvious from figure 3(a) that HA prepared from calcined egg shell in sucrose medium (H<sub>1</sub>Su-550) has well defined rod shape morphology and more segregated structure. The nanorod like morphology in the powder changed to a more equiaxed shape (spherical morphology) after sintering at 900 °C as shown in figure 3(b)

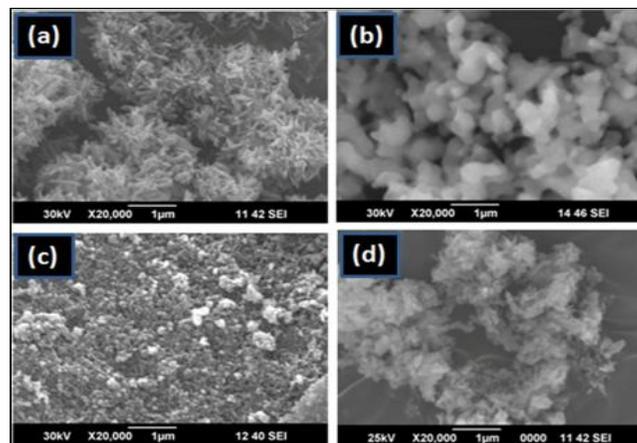


Figure 3: SEM photographs of (a) H<sub>1</sub>Su-550, (b) H<sub>1</sub>Su-900 (c) H<sub>2</sub>Su-550 (d) H<sub>1</sub>W-550

The increased size and spheroid morphology of the HA particles in the sample H<sub>1</sub>Su-900 in figure 3(b) may be explained by nucleation–aggregation–agglomeration growth mechanism theory explained by Randolph and Larson 1986 and Rodriguez et al 1998 [31, 32].

The sample H<sub>1</sub>W-550 in figure 3(d) shows agglomeration, but here also the rod shape is retained. It is obvious from the figure that the rods are irregular and highly agglomerated and even form lumpy masses .

The HA prepared from uncalcined egg shell H<sub>2</sub>Su-550 in figure 3(c) seems to exhibit a more or less spherical morphology. This is an undesirable morphology as far as bone implants are aimed at. The rod shaped HA mimics the natural bone mineral more closely than spherical one, and the resorbability of HA can be developed by improving the degree of crystallinity and reducing the grain size to nano level [33]. So calcined eggshell and sucrose medium could be optimized to be the apt precursor and environment to continue HA synthesis. Hence the sample H<sub>1</sub>Su-550 seems to be a better choice as a biomaterial for orthopaedic applications.

### 3.4. TEM Analysis

TEM studies (figure 4) confirmed the formation of rod shaped HA for the sample H<sub>1</sub>Su-550. The particle size of the powder was found to be less than 25 nm. The TEM images substantiated the role of sucrose as a dispersing agent in producing uniformly distributed rod shaped apatite.

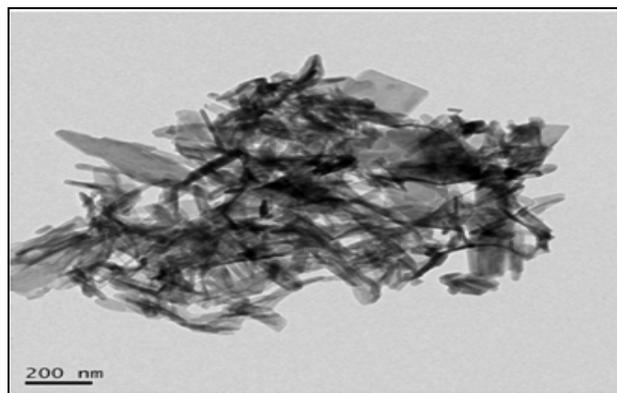


Figure 4: TEM photograph of H<sub>1</sub>Su-550

### 3.5. Elemental Analysis

The elemental composition and purity of prepared H<sub>1</sub>Su-550 was confirmed from EDX patterns and the data was well supported by ICP-AES. EDXS pattern (Figure 5) clearly showed the presence of Ca, P and O as expected for HA and also traces of carbon which was due to the incorporation of CO<sub>3</sub><sup>2-</sup> as observed in the FTIR analysis.

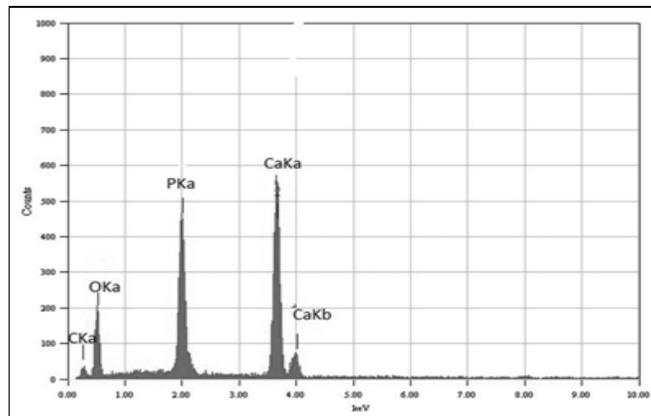


Figure 5: EDXS pattern of H<sub>1</sub>Su-550

ICP-AES data confirmed the presence of traces of Mg, Sr, Na apart from calcium and phosphorus (Table 3) which was derived from eggshells indicating the role of these ions in biomineralisation.

Element	Ca	P	Mg	Na	Sr
Weight %	34.124	15.343	0.333	0.393	0.093

Table 3: The elemental composition (wt/ wt%) of H<sub>1</sub>Su-550, using ICP-AES

#### 4. Conclusion

A simple, low cost, eco-benign method for the synthesis of nanohydroxyapatites of varying particle size, morphology and degree of crystallinity could be developed from egg shell waste. Nano HA of 19 nm diameter (spherical) were obtained when uncalcined egg shell stood as calcium precursor in sucrose medium. HA prepared from calcined egg shell in sucrose medium had well defined rod shape with 16 nm width at a final heating temperature 550 °C. When temperature raised to 900 °C the shape of HA changed from well defined rods to spheroid and the structure collapse evidenced from increase of size to 31 nm with a higher degree of crystallinity. HA prepared from calcined egg shell in water medium had an agglomerated and more amorphous structural features with particle width of 27 nm. The change of medium from water to sucrose increased the degree of crystallinity and reduced the size of prepared HA samples. SEM and TEM images reconfirmed the role of sucrose in obtaining uniformly shaped and well aligned nano HA rods. Thus the present work highlights the effect of the nature of the calcium source, temperature, and the medium of preparation on the size, crystallinity and morphology of the HA samples. Since the method promotes the extensive use of waste egg shell this will contribute to minimize the pollution especially in the areas of hatchery and catering units.

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