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Conversion of Ostrich Eggshells (*Struthio camelus*) to Calcium Phosphates

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Abstract
The exceptional progress made in orthopaedic and dental applications have increased the demand of calcium phosphate bioceramics due to their chemical similarities to the inorganic component of hard tissues. Low cost production of calcium phosphate bioceramics could be achieved by using pure natural biogenic materials by relatively simple methods. In this study calcium phosphate powders were produced from ostrich (*Struthio camelus*) eggshell powder at a moderate temperature of 80°C by relatively simple process of low temperature heating by hot plate (HP) and hot platting while agitation with ultrasonication (HPUS) hence introducing mechanical activation. The product structure and compositions were studied with FTIR, SEM, DTA/TCA, XRD and ICP techniques. The results showed that calcium deficient hydroxyapatite and dicalcium phosphate were obtained from HP and HPUS methods. Poorly crystalline calcium deficient hydroxyapatite was converted into whilockite after calcining at 800°C. The results suggest that this low cost and relatively simple method is efficient to easily produce calcium phosphate powders from adequately feed controlled farms to obtain pure uncontaminated eggshells for a range of biomedical applications.

Keywords: Calcium phosphates, ostrich, *struthio camelus*, mechanochemical, characterisation

1. Introduction

Calcium phosphate-based bioceramics have attracted much interest due to their high biocompatibility, ability to be used as synthetic osteoconductive scaffolds in bone and hence numerous clinical applications [1]. By definition, bioceramics can be grouped according to their bioactivity and resorption after implantation within the body into three categories such as bioinert ceramic, (alumina); bioactive ceramic, (hydroxyapatite (HAp), Bioglass® and A-W glass); while bioactive ceramic (by low crystalline or amorphous HAp, tri-calcium phosphate (α and β –TCP), tetracalcium phosphate (TTCP) and octacalcium phosphate (OCP) [2]. Considerable research efforts have been focused on the development of efficient and cost effective methods to produce calcium phosphate with apatite structure from biogenic natural materials. Different natural materials composed of calcium carbonate with unique architecture like sea coral [3], sea shells such as sea urchins [4], nacre [5], Mediterranean mussel [6], land snails [7], cuttlefish, and eggshell [8] which all have been reported to have potential to be converted to calcium phosphate materials for biomedical applications. Avian eggshells have been used in biomedical applications either direct as a bone substitute for maxilla-facial surgery [9] or as a starting materials for calcium phosphate bioceramics productions [10]. Eggshells of giant birds like ostrich (*Struthio camelus),* extant emu and extinct moa (*Dromaius novaehollandiae)*, rhea (*Rhea Americana*), kiwi (*Apteryx australis*), and cassowary (*Casuarius casuarius)*, as well as elephant bird (*Aepyornis maximus)* have been ignored due to possible contaminations in-spite of their potentials in biomedical field. In this century where proper artificial growing and farming methods and controlled diets are possible, artificial farming can produce high purity raw materials.

Among giant birds, *Struthio* is used as a palaeoproxy in palaeoenvironmental reconstructions as well as a

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bone substitute in biomedical field [9]. Yannicke *et al.* [11] have investigated microstructure and composition of the layers of *Struthio* eggshells and suggested that results are similar to those obtained on chicken eggshells, and proposed the active role of sulfated acidic polysaccharides in biomineralization processes of carbonate skeletons. Megan and Maxwell [12] reviewed the results of recent proteomic and genomic analyses of eggshell matrix proteins and draw attention to the impact of these data on current understanding of eggshell formation and function. They proposed the dual role of eggshell-specific matrix protein (mineralization/antimicrobial protection) that can play.

Several synthesis routes for HAp and β –TCP have been reported in the literature. Among them, the main two are the wet chemical and solid-state reaction methods [13, 14]. Alternative techniques like mechano-chemical, electrospray, hydrothermal, microwave heating just to mention a few, have been reported previously [15, 16]. Mechano-chemical technique is simple, cost effective method to synthesize inorganic compounds [17]. During mechano-chemical synthesis, disperse particles are formed due to the pulverization and hence increased surface area of the reagents and chemical interaction between these nano and meso size reagent components. It was revealed in the past that particles of the reaction products are formed as two-dimensional nuclei at points of contact between components and then grow. Reaction product particle size can easily be controlled by varying the temperature, agitation and reaction time of subsequent mixture. It was suggested that numbers of compounds have been synthesized in the nanodispersed state by mechanical activations of the reaction mixture [18, 19]. Hassane *et al.* [20] successful synthesized HAp from dicalcium phosphate dehydrate and calcium oxide by dry mechanochemical method and suggested that this technique is one of the other possible means to synthesize HAp with an expected molar calcium-to-phosphorous (Ca/P) ratio 1.67± 0.005. However many factors influence the particle size and morphology and the process of step by step crystallisation and growth is not clearly explained or understood.

This research is focused on the production and characterizations of calcium phosphate bioceramic materials, HAp and β –TCP, by wet mechanochemical synthesis technique from *Struthio camelus* eggshell to understand these formation mechanisms. It has been previously reported that hydroxyapatite powder can be synthesized using waste hen eggshells as calcium source by a straightforward hydrothermal method [21] (with some retained fraction of other calcium phosphate compounds), by attrition milling [10] and chemical methods [8, 22]. The challenges in these methods are reaction/milling time and the purity of the final products.

Our main aim in this research is to compare two synthesis methods by employing mechanical activation under moderate reaction conditions of temperature to produce single phase calcium phosphate materials from ostrich eggshell and understand the morphological and crystallographic changes during the process and following calcination treatments.

2. Experimental

2.1. Materials

Ostrich eggshell were obtained from Turkey while phosphoric acid and other chemicals used were obtained from Sigma Aldrich Ltd., Sydney, Australia.

2.2. Methods

2.2.1. Conversion of Ostrich eggshell powder to calcium phosphate.

The thermal conversion of the $CaCO_3$ to CaO of *Struthio camelus* was determined by differential thermal and thermogravimetric analysis (TG-DTA, SDT 2960, TA Instruments, NewCastle, DE, USA). Sample weight of 30 - 40 mg was used during analysis under circulating air environment with a heating rate of 10^{o}C/min from room temperature to 1100^{o}C. The samples were first cleaned by using 5% NaClO [3] then grounded within a ball mill (46 rpm, 2 h), sieved through 75-100μm sieve and then cleaned again by NaClO before conversion.

Two conversion methods were employed: First, eggshell powders were suspended in distilled water in a well washed beaker, heated on a hot plate (HP) at 80^{o}C under no mechanical stirring. Then required amount (equivalent to the $CaCO_3$ amount to convert tostoichiometric molar ratio for HAp) of H_3PO_4 was added drop wise to the solution.

The second method for the ultrasonic stirring conversion (HPUS) was performed by suspending the raw powder in distilled water in a well-washed beaker under mechanical stirring equipped with ultrasonic probe with the temperature set to 80^{o}C. Then, the equivalent amount of H_3PO_4 was added drop-wise to the solution. After conversion, the mixtures were filtered (pore size, 2.5 μm filter paper) and then dried in an oven at 100°C for 24 h then calcined according to the required phase (s) at 400 and 800^{o}C

2.2.2. Characterisation.

The morphological analyses of eggshell and powders before and after conversion were performed using Scanning Electron Microscopy (SEM) (ZEISS Supra55VP, Zeiss, Germany). Samples were fixed by mutual conductive adhesive tape on aluminium stubs and coated with carbon using a sputter coater. Images were taken at various magnifications at acceleration voltages of 20 kV.

Phase analysis of the synthesized powders of calcium phosphates were conducted by X-ray powder diffraction using Siemens D5000 X-ray Diffractometer (Siemens, Bayswater, Australia) employing CuK_α radiation (λ=0.15418 nm) with detector (X'celerator). The diffractometer was operated at 45 kV and 40 mA at a 2θ range of 10–70° employing a step size of 0.01 and a 4 s exposure.

The synthesized calcium phosphate powders were ground in an agate mortar and thoroughly mixed with KBr (FTIR Grade). Three milligrams of powder sample was mixed with 300 mg of KBr powder (1% w/w). Transparent pellets were prepared in a stainless steel die by applying a uniaxial load of 6.89 MPa pressure (Carver press). The FTIR spectra were collected using a Nicolet, Magna-IR 6700 Spectrometer FTIR (Thermo Fisher Scientific, Madison USA) in the range 2200–400 cm^{-1}.

Powders were digested to quantify calcium, phosphorous, magnesium and sodium ions by inductively coupled plasma-mass spectroscopy (ICPMS). An Agilent Technologies 7500ce series ICP-MS (Agilent Technologies, Japan) was used with sample introduction via a micromist concentric nebuliser (Glass expansion). The ICP operating parameters and the lens conditions were selected to maximize the sensitivity of a 1% HNO_3:HCl solution containing 1ng/ml of Li, Co, Y, Ce and Tl. Calibration standards were prepared in 1% nitric acid. Approximately 0.0005g of sample was digested with 0.25 mL of HNO_3. The samples underwent a further 1:100 dilution with a 1% nitric acid solution before ICP-MS analysis.

3. Results

Amount of calcium carbonate in ostrich eggshell was determined by thermogravimetric analysis in TG-DTA. Figure 1 shows the weight loss of ~ 42.5% due to thermal decomposition of calcium carbonate to calcium oxide and carbon dioxide at ~ 780°C which is the main composition of *Struthio camelus*. The weight loss due to organic matter was ~ 2.6 %.

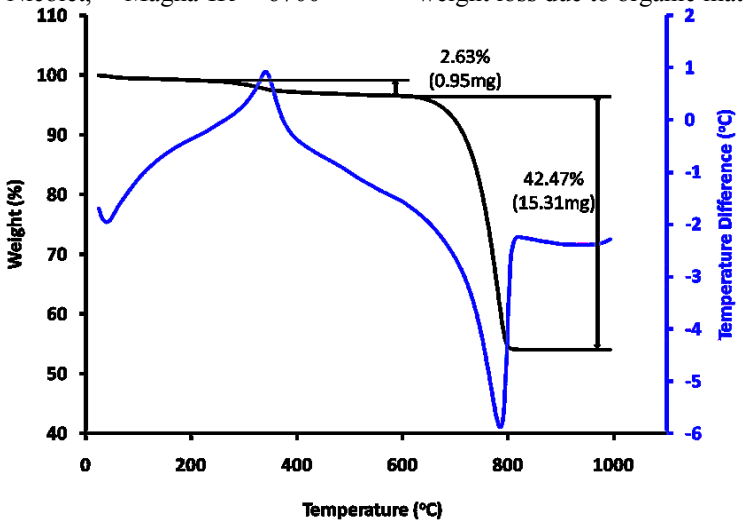


Fig. 1: TG/DTA of raw ostrich eggshell

Table 1. ICP MS for converted and unconverted ostrich egg shell to calcium phosphate

	Calcium (mg/g)	Sodium (mg/g)	Magnesium (mg/g)	Phosphorous (mg/g)	Strontium (mg/g)	Barium (mg/g)
***Struthio camelus* eggshell**	6.01 ± 0.01	2.85 ± 0.01	0.30 ± 0.01	0.10 ± 0.02	0.043 ± 0.002	0.008 ± 0.002
HP_Conversion	5.80 ± 0.01	2.77 ± 0.01	0.29 ± 0.02	12.79 ± 0.02	0.028 ± 0.005	0.008 ± 0.003
HPUS_Conversion	4.34± 0.01	2.27 ± 0.02	0.20 ± 0.01	9.31 ± 0.001	0.018 ± 0.006	0.006 ± 0.003

Inductively coupled plasma mass spectrometry (ICP-MS) measurements of selected element contents (Table 1) reveal that *Struthio camelus* eggshell has trace elements of low Mg, P, Sr and Ba contents. On the other hand, *Struthio camelus* eggshell exhibits high Ca (around 6 mg/g) and high Na (around 2 mg/g). Converted products from the two methods exhibit around the same calcium content (around 6 and 4 mg/g) and high content of phosphorous (around 13 and 9 mg/g) for HP and HPUS respectively. Sodium, magnesium, strontium and barium decreased marginally after conversion by HP but magnesium and strontium decreased significantly after conversion by HPUS.

The FTIR spectrum of raw ostrich egg shell (ROES) and converted ostrich eggshell powders in Figure 2a&b. Relevant vibrations of HAp, whitlockite, di-calcium phosphate amorphous (DCPA) and calcite for the powders obtained from HPUS and HP conversion methods have been found. For the samples calcined at 400°C (Figure 2a) show the vibrations of HAp and calcite while for 800°C revealed the vibrations of HAp, TCP and DCPA. Vibration band at 634 cm^{-1} is referable to structural OH groups, (OH) stretching and vibration modes at the HAp crystalline surface or at the crystallites. Presence of $[PO_4]$ groups, characteristic to HAp structure is depicted by vibration band at 472 cm^{-1} as a bending vibration of $[PO_4]$ v_2. In the range 700 – 500 cm^{-1} HAp has medium intensity vibration bands 602, 563 cm^{-1} and a shoulder at 634 cm^{-1}. The Whitlockite bands at 563 cm^{-1} and 613 cm^{-1} correspond to the symmetric and asymmetric deformation modes of $[PO_4]$ v_4. The band at 454 cm^{-1} and 634 cm^{-1} are assigned to the vibrational mode of $[PO_4]$ v_2 of whitlockite and of hydroxyl group in HAp respectively [23]. The broad band in the range 1040 – 1100 cm^{-1} corresponds to a band characteristic to $[PO_4]$ v_3 asymmetric stretching modes. The presence of calcite in powders is attributed by the absorption band at 876 cm^{-1} which corresponds to $[CO_3]$ v_2 vibration mode. Absorption band at 712 cm^{-1} is due to the in-plane bending mode of $[CO_3]$ v_4. Absorption maximum of $[CO_3]$ group at 876 cm^{-1} can also prove that AB-type PO_4 and OH groups substitution in the structure of HAp. The presence of DCPA is attributed by the vibration bands at 1410 cm^{-1} and 525 cm^{-1} (v_3 PO_4), 892 cm^{-1}, and 428 cm^{-1} (v_2 PO_4).

The powder X-ray diffraction patterns of the *Struthio camelus* eggshell and powders obtained by HP and HPUS conversion methods after calcination are shown in Figure 3a&b**.** The spectra provide a clear evidence of the appearance of crystalline phases from both methods. *Struthio camelus* eggshell powder gave a strong reflection centered at 2θ = 29° which correspond to the (1 0 4) reflection of calcite (Figure 3a). This phase is also present as the secondary phase in the converted eggshell by both HP and HPUS conversion methods calcined at 400°C. The reflection observed at 2θ = 26° and 2θ = 32° for the products calcined at 400°C, are the strongest reflections of apatite phase and are attributed to the (0 0 2) and (2 1 1) reflections of $Ca_{8.86}$ $(PO_4)_6$ $(H_2O)_2$ (JCPDS card no. 05-1199) respectively. The x-ray diffraction patterns of the powder obtained by HP and HPUS conversion methods and calcined at 800°C (Figure 3b), show peak spacing consistent with crystalline whitlockite $Ca_3(PO_4)_2$ (JCPDS 09-0169). Whitlockite is further confirmed by the strong reflections observed at 2θ = 28°, 31° and 34° which are the reflections attributable to the (2 1 4), (0 2 10), (2 2 0) reflections respectively. Traces of HAp was also observed in x-ray diffraction patterns from samples calcined at 800°C with reflectons

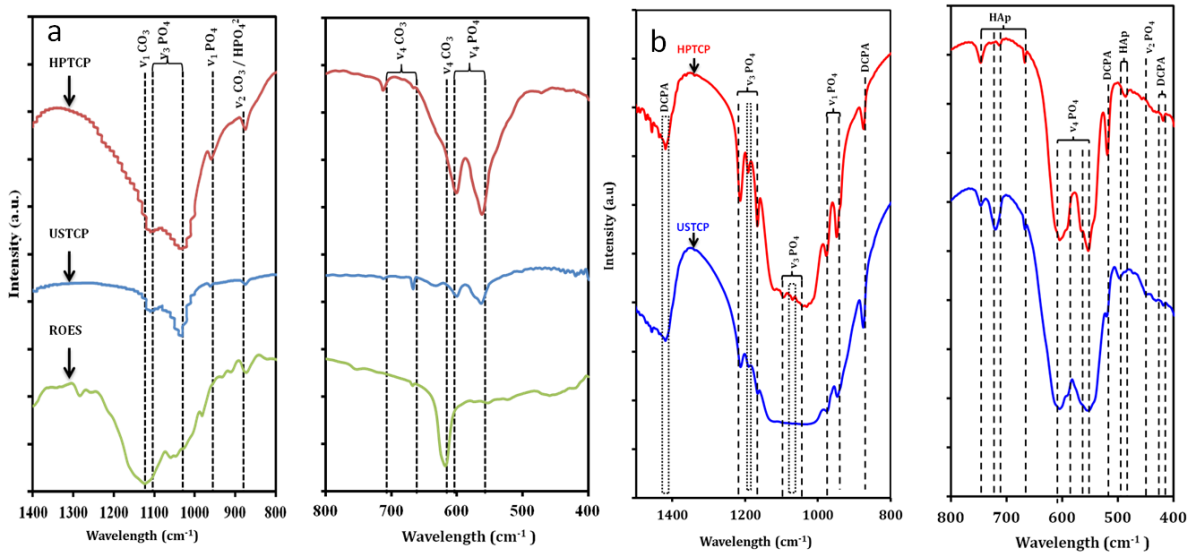


Fig. 2: FTIR spectra **a)** Calcined at 400°C for 3 hrs **b)** Calcined at 800°C for 3 hrs

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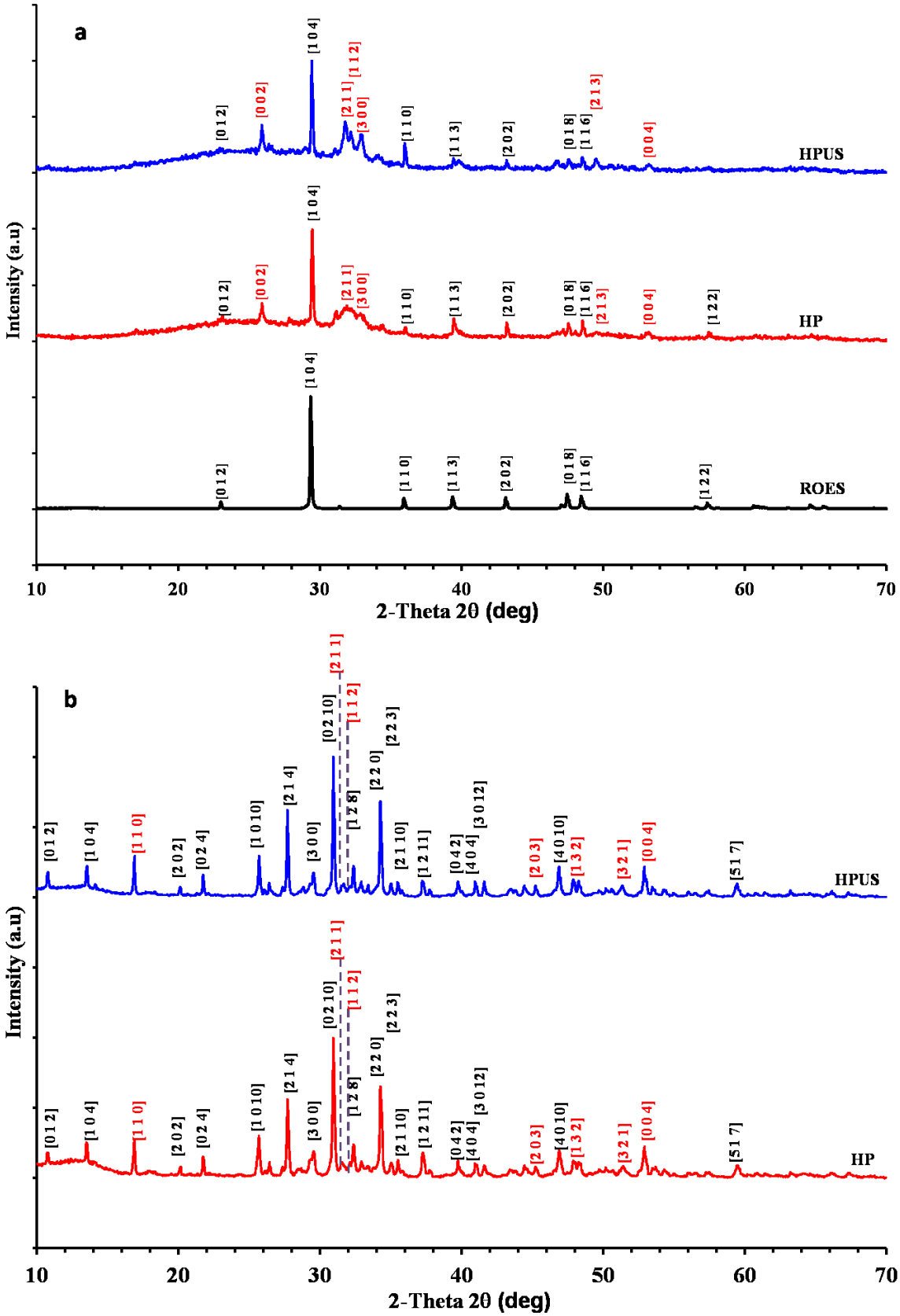


Fig. 3: XRD spectra of converted powder and calcined for 3 hrs at **a)** 400^{o}C showing the presence of Calcite and CA-Deficient Hydroxyapatite **b)** 800^{o}C showing the presence of Whitlockite and Ca-Deficient Hydroxyapatite

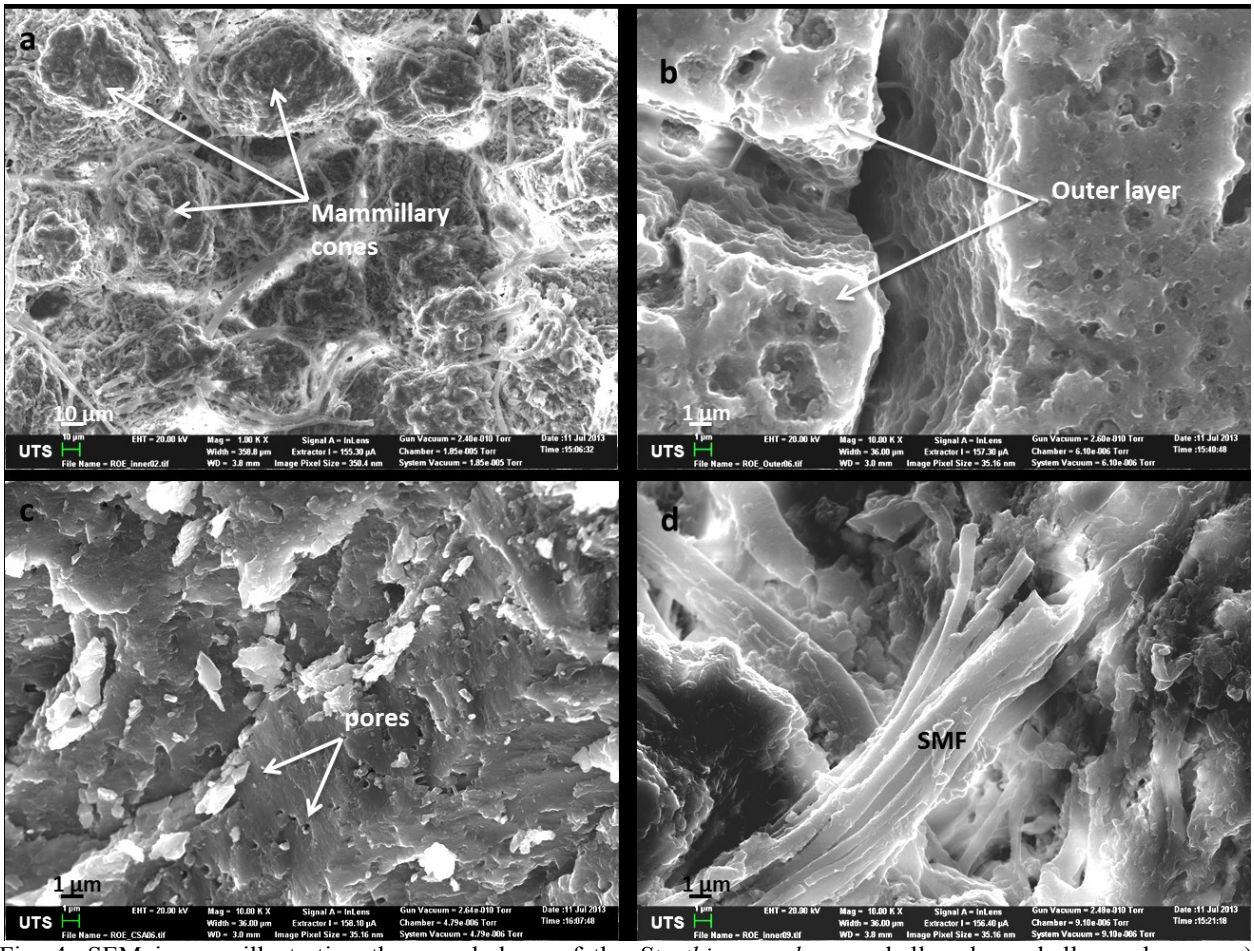


Fig. 4: SEM images illustrating the morphology of the *Struthio camelus* eggshell and eggshell membranes. a) Cleaned inner surface showing the mammillary cones. b) Outer cleaned layer showing outer prismatic layer and a thin organic cuticle covers the outer surface. c) Fractured *Struthio* eggshell (cross sectional) showing the presence of discrete pores d) High magnifications of inner surface showing shell membrane fibres (SMF) to reveal their interwoven and coalescing nature.

Struthio eggshell is composed of several organic and calcite layers that are classified into four main layers from the inner to the outer shell namely fibrillar organic membranes, the mammillary layer, the palisade layer, and the outer prismatic layer [11]. Figure 4 shows the different morphology of the eggshell. *Struthio* egg shell consists mainly of calcite which is the most stable polymorph of calcium carbonate, and extends from the inner mammillary cone layer (Fig. 4a) through the central palisades (not shown) and the outer vertical crystal layers [24]. The outer shell (Fig. 4b) which consists of the thin organic cuticle is composed of glycoproteins, polysaccharides, lipids and inorganic phosphorus including HAp crystals in its inner zone [25]. The discrete pores shown on the fractured eggshell (Fig. 4c) permit the diffusion of metabolic gases and water vapour to allow proper embryonic development [26]. The inner uncalcified layer shown in Fig. 4d reveals the network of fibres that envelops the albumen.

4. Discussions

Calcium phosphate bioceramics are the main constituents of bones and teeth and alternative methods for production from natural biogenic materials is of great interest to both scientists and industry. Production of HAp from waste biogenic materials like eggshells have been reported in the literature [8, 10, 21, 22]. The only common thing reported in literature, despite the significant disparity in the final products, starting materials or methods, is the formation of useful calcium phosphate materials with numerous applications such as bone substitute and regeneration, food additives, drug delivery systems, just to mention few. In this investigation, biomedical products were obtained from two different synthesis methods of calcium phosphate materials using ostrich eggshell and orthophosphoric acid. The FTIR spectra, Figure 2b and XRD pattern, Figure 3b of the final products obtained from HP and HPUS methods suggested the formation of whitlockite

material ($Ca_3(PO_4)_2$) with small amount of calcium deficient hydroxyapatite ($Ca_{8.86}(PO_4)_6(H_2O)_2$). Different final products were obtained when the converted powders from HP and HPUS were calcined at low temperature of 400°C. FTIR (Figure 2a) and XRD (Figure 3a) results suggested that calcium deficient hydroxyapatite ($Ca_{8.86}$ $(PO_4)_6(H_2O)_2$)was obtained with calcite ($CaCO_3$) as a secondary unconverted phase.

The morphologies of products before and after calcined at 400°C and 800°C are shown in Figure 5. It has been shown that before calcination the morphology of products from HP (Fig. 5a) and HPUS (Fig. 5b) have the thin ribbons/blades shape crystals which are consistent to the morphology of HAp. After heat treatments, the morphology was converted to platelets at 400°C, with well dispersed flake like structure and interconnective porosity (Fig. 5c&d), while after 800°C heat treatment densification occurred with smaller plates with much smaller amount porosity and finer structure (Fig. 5e&f).

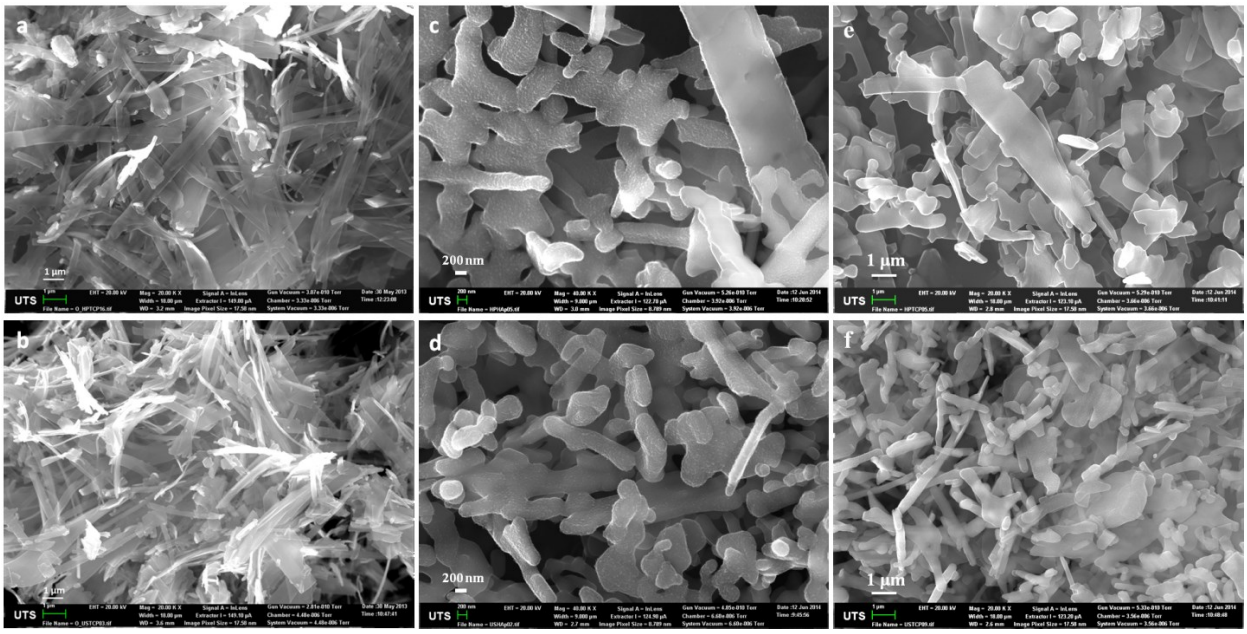


Fig. 5: SEM image showing the morphology converted products of *Struthio camelus* eggshell powder by HP and HPUS before calcinations (a&b), calcined at 400°C (c&d) and at 800°C (e&f).

Table 2: Phase quantifications of powder calcined at 400°C for 3 hrs

JCPDS Card No.	Phase name	Formula	HP		HPUS	
			PIR	%W	PIR	%W
05-0586	Calcite	$CaCO_3$	2.0	40.1	2.0	31.4
05-1199	Ca-Deficient Hydroxyapatite	$Ca_{8.86}$ $(PO_4)_6(H_2O)_2$	0.8	34.6	0.8	40.4
Global amorphous - DCPA			0.8	25.3	0.8	28.2

Table 3: Phase quantifications of powder calcined at 800°C for 3 hrs

JCPDS **Card No.**	**Phase name**	**Formula**	**HP**		**HPUS**	
			PIR	%W	PIR	%W
9-0169	Whitlockite	$Ca_3(PO_4)_2$	1.0	73.7	1.0	77.9
82-1943	Hydroxyapatite	$Ca_{8.86}(PO_4)_6(H_2O)_2$	0.8	11.0	0.8	9.7
Global amorphous - DCPA			0.8	15.3	1.0	12.4

XRD quantifications of the resulted phases was carried out by using Xpowder 12 software fon full profile quantitative analysis of components (using 'Dirac' patterns) and convolution and PIR scale factor. It has been shown that after heat treatment at 400°C as shown in (Table 2) about 40 % in HP and -31 % in HPUS of unconverted calcite was retained in the samples. Calcium deficient hydroxyapatite (HAp) of about 34% and 40 % was quantified in the final products from HP and HPUS respectively. It has also been revealed that there is an increase of about 28% yield of HAp when an increase in mechanical activations (mechanical stirring and ultrasound) HPUS was utilized. Both conversion methods produced some amorphous di-calcium phosphate materials (DCPA). The reaction schemes of conversion process is similar to one we suggested in our earlier publication [27].

X-ray analysis revealed that calcium deficient hydroxyapatite was converted to tri-calcium di-phosphate (whitlockite) when calcined at 800°C. Table 3 shows the absence of calcite after 800°C treatment which suggests that its decompositions products were consumed in the formation of whitlockite.

5. Conclusions

Struthio camelus eggshell was successfully converted into mainly whitlockite by simple heating and mechanochemical conversion methods under moderate temperature. The effects of morphology and structure of these ceramics have also been studied. The results suggest that mechanochemical conversion method could be an effective method to produce bioceramic materials from natural biogenic materials within reasonable time periods. An increase in mechanical activations (mechanical stirring or ultrasound) at temperature 80°C increases the yield of Ca deficient defect HAp by 17%. On the other hand 5.6% yield increase of whitlockite was observed after calcination at 800°C as revealed by XRD of final products from HP and HPUS conversion methods. It is proposed that a range of other natural sources of calcium could also be used to produce calcium phosphate bioceramics using mechanochemical conversion method.

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