Ultrasound Assisted Green Economic Synthesis of Hydroxyapatite Nanoparticles using Eggshell Bio-waste and Study of Mechanical and Biological Properties for Orthopaedic Applications

Vijay H. Ingole[a,b], Kamal Hany Hussein[c], Anil A. Kashale[a], Kalyani Ghule[a], Tomaž Vuherer[b], Vanja Kokol[b*], Jia-Yaw Chang[d], Yong-Chien Ling[e], Aruna Vinchurkar[f], Hom N. Dhakal[g] and Anil V. Ghule[a,h,*]

[a]*Department of Nanotechnology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad 431004, Maharashtra, India*

[b]*Faculty of Mechanical Engineering, University of Maribor, Maribor 2000, Slovenia*

[c]*Stem Cell Institute and College of Veterinary Medicine, Gangwon National University, Chuncheon, Gangwon 200-701, South Korea,*

[d]*Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 10607, Taiwan*

[e]*Department of Chemistry, National Tsing Hua University, Hsinchu 30013, Taiwan*

[f]*Department of Biophysics, Government Institute of Science, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad 431004, Maharashtra, India*

[g]*School of Engineering, University of, Portsmouth, Hampshire PO1 3DJ, United kingdom*

[h*]*Department of Chemistry, Shivaji University, Kolhapur 416004, Maharashtra, India*

E-mail: anighule@gmail.com; avg_chem@unishivaji.ac.in

**Corresponding Author: Anil Vithal Ghule; E-mail: anighule@gmail.com*

Abstract

Nanostructured hydroxyapatite (HAp) is most favorable candidate biomaterial for bone tissue engineering because of its bioactive and osteoconductive properties. Herein, we report for the first time ultrasound assisted facile and economic approach for the synthesis of nanocrystalline hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) using recycled eggshell bio-waste referred as EHAp. The process involves the reaction of eggshell bio-waste as a source of calcium and ammonium dihydrogen orthophosphate as a phosphate source. Ultrasound-mediated chemical synthesis of hydroxyapatite (HAp) is also carried out using similar approach wherein commercially available calcium hydroxide and ammonium dihydrogen orthophosphate was used as calcium and phosphate precursors, respectively and referred as CHAp for better comparison. The prepared materials were characterized by X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy to determine crystal structure, particle morphology, and the presence of chemical functional groups. The nanocrystalline EHAp and CHAp were observed to have spherical morphology with uniform size distribution. Furthermore, mechanical properties such as Vickers hardness, fracture toughness, and compression tests have been studied of the EHAp and CHAp samples showing promising results. Mechanical properties show the influence of calcination at 600^{o}C EHAp and CHAp material. After calcination, in the case of EHAp material an average hardness, mechanical strength, elastic modulus and fracture toughness were found 552 MPa, 46.6 MPa, 2824 MPa and 3.85 MPa·$m^{1/2}$ respectively, while in the case of CHAp 618 MPa, 47. 5 MPa, 2071 MPa and 3.13 MPa·$m^{1/2}$. In vitro cell studies revealed that the EHAp and CHAp nanoparticles significantly increased the attachment and proliferation of the hFOB cells. Here, we showed that EHAp and CHAp provide promising biocompatible materials that do not affect the cell viability and proliferation with

enhancing the osteogenic activity of osteoblasts. Moreover, hFOB cells are found to express Osteocalcin, Osteopontin, Collagen I, Osteonectin, BMP-2 on the EHAp and CHAp bone graft. This study demonstrates the formation of pure nanocrystalline HAp with promising properties justifying the fact that the eggshell bio-waste could be successfully utilized for the synthesis of HAp with good mechanical and osteogenic properties. These finding may have significant implications for designing of biomaterial for use in orthopedic tissue regeneration.

Keywords: Eggshell, biocompatibility, hFOB cells, mechanical properties, ultrasonication.

1. Introduction

The modern era is experiencing remarkable development in advanced synthetic approaches of novel materials and biomaterials of societal significance with the advent of nanotechnology. Similarly, the urge for development of biomedical devices, tissue grafting and implants for use in tissue engineering applications is growing and is the need of the time[1]. Loss of bone tissue as a result of diseases, trauma, and injury are on the rise among the population which affects their quality of life at a significant socio-economic cost. Bone tissue transplantation is the second most common therapy, with 2.2 million bone grafts being performed annually worldwide[1-3]. However, bone grafts from natural sources have their own limitations, thus there is need to develop biosynthetic materials to mimic the natural biomaterial bone grafts. Natural biomaterials like bone substitutes from a bovine source, cardiovascular prostheses of biological origin, wound dressings made of biologically derived calcium alginate, collagen, chitin etc., have recently become prominent synthetic biomaterials by providing an abundant source for novel biomedical applications[4]. It is known that the mineral phase of bone tissue occupies about 69% of its total weight, in which the hydroxyapatite (HAp), with chemical formula, is $Ca_{10}(PO_4)_6(OH)_2$, is the most significant[5]. Hydroxyapatite (HAp), the inorganic constituent of bone and hard tissues, is one of the most widely used biomaterials for reconstruction of the skeleton due to its excellent bioactivity and biocompatibility with the human body[4,6]. The close chemical similarity of HAp to natural bone has led to extensive research efforts to use synthetic HAp as a bone substitute, biocompatible coatings on medical devices and implants and replacement in biomedical applications[7]. Apart from this, HAp is also being explored for various industrial applications such as catalyst support, liquid chromatographic columns, lighting materials, powder carriers, sensors, ion conductors, retardant of cancer cells and

drug delivery agent, etc[8-10]. Commercially available HAps are expensive due to the use of high purity reagents[4]. Furthermore, the HAp derived from natural materials such as bovine bone, fish bone or coral has the advantage that they inherit some properties of the raw materials such as the pore structure, carbonated HAp etc. However, problems do arise due to the variability of physical and chemical properties of the raw material[4]. On the other hand, the eggshell represents 11% of the total weight of the egg and is composed predominantly of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%)[4,11,12]. It is estimated that the world's egg production was approximately 6.37 x 10^7 tons in 2010 (Global poultry trends, 2010) which ultimately results in the generation of eggshell bio-waste, recycling of which is a great challenge which is yet to be addressed satisfactorily. Taking into account that the shell occupies about 11% of the weight of each egg, the amount of eggshells produced in the world in the last year ascends to about $7x10^6$ ton. This material is basically useless after the production of eggs and egg derivatives. Moreover, in many cases, once the egg is used, manufacturers store the bio-waste and is then discarded, which contributes to environmental pollution due to the presence of adhered organic-bioorganic compounds in these wastes, producing bad odor and give rise to health challenges.[5] Though these are occasionally used as a fertilizer due to their high content of calcium and nitrogen, their absorption as fertilizers and nutrients is doubtful as this is rarely are used as part of the diet of some animals and chickens, however, the benefits are scarce. Furthermore, HAp materials which are prepared from bovine and human resources chemical processes such as treating them with acids are not safe as some diseases can survive all controlled processes. For example prions, a small proteinaceous infectious particle can survive such processes although high-temperature calcinations above 850^{o}C could solve this problem of transmission of diseases[13]. Realizing the situation, utilizing this biowaste, namely

eggshell, the cost of high-quality calcium source for preparation of HAp can be avoided and at the same time recycling of these eggshell waste materials would not only reduces the cost of preparing HAp but also promotes environmentally benign process[4,5].

Natural bone minerals are nanostructured non-stoichiometric HAp of dimensions 20 nm in diameter and 50 nm long with the substitution of ions such as magnesium, fluoride, and carbonate[14,15]. From bionics viewpoint, synthetic apatite to be used for repairing damaged hard tissues, are expected to have characteristics closer to those of biological apatite in both composition and structure[16]. Nanocrystalline HAp has been shown to exhibit enhanced bioactivity[17]. Calcium salts such HAp[18], β-tricalcium phosphate (β-TCP) and calcium carbonate ($CaCO_3$)[19] can also be incorporated to mimic the inorganic component of native bone and to improve the osteoconductivity of the material construct[6]. In recent years, significant research effort has been devoted to developing the preparation and morphology control methods of HAp. HAp with various morphologies have been synthesized by means of solid-state reaction[20-22], sol–gel method[23,24], template-directed method[25,26], hydrothermal method[27,28], and microemulsion[29,30] or emulsion technique[31]. Indeed, morphology and properties depend strongly on the synthesis approach and conditions employed. Some studies demonstrated eggshell derived preparation of hydroxyapatite by high-temperature processing[4,11], which could be relatively expensive practice. However, herein this work nanocrystalline hydroxyapatite (HAp) is accomplished by a precipitation method mediated with ultrasonic irradiation. In recent years, there have been manifold reports of ultrasonically assisted synthesis of hydroxyapatite and it is a promising technique to produce nanostructured HAp at the cutting edge quality standards. The ultrasonic route allows producing nanocrystalline HAp as readily as modified particles, e.g. core-shell nanospheres and composites[32-35].

Thus, in this study, a unique way yet simple method to convert eggshell bio-waste into nanocrystalline HAp by ultrasonication mediated processing for the synthesis of EHAp has been presented for the first time to the best of our knowledge. The physicochemical characterization of the nano-HAp powder is also carried out. Since CHAp can also be synthesized by using chemicals by a similar method which was used to the synthesis of EHAp. To highlight the advantages of using eggshell as a calcium source for the synthesis of HAp (EHAp), the results were compared with HAp (CHAp) prepared using synthetic calcium hydroxide by similar ultrasonication process. To check the purity and crystallinity EHAp and CHAp are calcined at 600 ^{o}C and named as EHAp600 and CHAp600, respectively. The successful synthesis of EHAp is confirmed using various characterization techniques.

2. Experimental

2.1 Materials

In this study, calcium oxide derived from eggshell is used as a calcium source and synthetic ammonium dihydrogen orthophosphate is used as phosphorus precursor. Ammonium hydroxide, ammonium dihydrogen orthophosphate, and hydrogen peroxide were obtained from Fisher Scientific, India. All the reagents procured were of analytical grade and were used without further purification. Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (Sigma-Aldrich, St Louis, MO, USA), Penicillin/streptomycin (Gibco, Grand Island, NY, USA), Fetal bovine serum (Hyclone, Logan, UT, USA), mM L-glutamine (Gibco, Grand Island, NY, USA), 3-(4,5-dimethylthiazol)-2-yl]-2,5-diphenyltetrazolium bromide (Sigma- Aldrich, St Louis, MO, USA), SPECTRA max® Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA), LIVE/DEAD® Cell Viability Assay (Invitrogen, USA), Fluorescence

microscope (Olympus, Tokyo, Japan), NucleoSpin kit (Machery-Nagel Gmbh& Co.KG, Germany), NanoDrop spectrophotometer ND-1000 (PeqLab, Erlangen, Germany), Go Script reverse transcriptase (Promega, Korea), TRIzol solution (Invitrogen, USA), TProfessional standard 96 gradient machine (Biometra, Goettingen, Germany), Ultraviolet transillumination (G:BOX F3, Syngene, Cambridge, UK), and 100-base pair DNA ladder (GeneRuler, Fermentas, Burlington, ON, Canada) were used for biological study.

2.2 Synthesis and Processing

Eggshells of boiled eggs were collected from vendors from the local market. In order to eliminate the adhered organic matter, the eggshells were crushed and washed with de-ionized (DI) water. After washing, the eggshells were ultrasonicated in deionized water and 25 vol% of reagent grade H_2O_2 for 1 h. The ultrasonic processing was performed using Ultrasonic bath (9L250N/D.T.C, PCI Analytics Pvt. Limited, India) operated at 230 V AC, 100 % power and at 50 kHz frequency. After ultrasonication, the eggshell were washed with DI water and dried in an oven for 2 h at 100 ^oC. After drying, the eggshells were ground and ball milled to obtain a fine powder of nanoparticles. Further, the eggshells were heated with an interval of 200 ^oC and calcined at 900 ^oC for 2 h in a box furnace to decompose organic matter and to convert the calcium carbonate to calcium oxide. The calcium oxide obtained from eggshells was weighed and dispersed in DI water and ammonium hydroxide and ultrasonicated for 1 h to form CaOH. Subsequently, hydroxyapatite (EHAp) was synthesized using sonochemistry method. In a typical synthesis of EHAp, 1.23 gm of CaOH obtained from eggshells was reacted with 1.15 gm of reagent grade ammonium dihydrogen orthophosphate corresponding to the stoichiometric ratio of Ca/P=1.67. CaOH and ammonium dihydrogen orthophosphate

were mixed and ground in a mortar and pestle. The ground mixture of CaOH and ammonium dihydrogen phosphate was dispersed in 50 mL DI water and heated on a magnetic stirrer with stirring for 15 min. After this, the solution was ultrasonicated for 1 h and then heated on magnetic stirrer to evaporate the water producing EHAp. The product was further ground in an agate mortar pestle to obtain fine nanoparticles of EHAp. A similar protocol was adopted for chemical synthesis of hydroxyapatite (CHAp) except for the use of synthetic reagent grade calcium hydroxide as a source of calcium for comparative study.

A small amount of both the samples were heated at 600 ^{o}C for 1 h to improve the crystallinity and purity and labeled as EHAp600 and CHAp600, respectively.

2.3 Characterization

The powder X-ray diffraction (XRD) measurements were carried out at room temperature using D8 ADVANCE BRUKER diffractometer with Cu $K\alpha_1$ radiation (λ=1.54056Å), operated at voltage 40 kV and 44 mA setting over a 2θ range from 10 to 90^{o} at a scanning speed of 1^{o} per min. The average crystallite sizes were estimated using the Scherrer's formula. The morphology of the synthesized particles was investigated at various magnifications from 5k to 200k by a field emission scanning electron microscope (FESEM, Hitachi-S4800, Japan operated at 20 kV) coupled with EDX. Prior to the FESEM observations, a small amount of powder were placed onto a conducting carbon tape placed on the FESEM specimen holder and sputter coated (using a Hitachi E1010 Ion sputter, Japan) with gold in order to minimize the charging effect resulting from the electron beam. Simultaneously, the elemental compositions of the samples were analyzed by using Energy Dispersive X-ray Spectroscopy (EDX). Nanostructures of the particles were investigated using transmission electron microscopy (TEM, HD-2300,

Hitachi, Japan) with an operating voltage of 200 kV. The particle size distribution study was performed using dynamic light scattering (DLS) measurement (Zetasizer Nano, Malvern Instruments Ltd, UK). The functional groups present in CaO, CaOH, EHAp, CHAp, EHAp600 and CHAp600 were ascertained by Fourier transform infrared spectroscopy (FT-IR). The FT-IR (CARRY 600 Series, Japan) spectra were obtained over the region 400–4,000 cm^{-1} using ATR technique with a spectral resolution of 4 cm^{-1}. Raman spectrophotometer (STR-150 series, Japan) with laser excitation source and an excitation wavelength of 532 nm was used for the analysis of the samples, for which, the samples were placed on a glass slide prior to the analysis. The laser beam was focused onto the sample surface and the Raman spectra of CaO, CaOH, EHAp, CHAp, EHAp600 and CHAp600 were analyzed.

2.4 Mechanical properties studies

Mechanical tests (Vickers's hardness, compression tests, and fracture toughness) were performed for EHAp, EHAp600, CHAp, and CHAp600. The HAp sample powders were used to prepare two types of cylindrically shaped pellets (100 and 200 mg) with a diameter of 5 mm without adding binder or plasticizer by using compression technique. The pellets were prepared by compression methods under controlled conditions in which force, crosshead velocity and displacement were controlled and measured by tensile testing unit (TTU 2002) of SMITWELD 1405 testing machine. To prepare the pellets the crosshead velocity was 0.05 mm/s and a maximum force applied was 15000 N. The pellets with 100 mg material weight were used for Vickers hardness test and fracture toughness test, while the pellets with 200 mg material weight were used for a compression test. The density of all the pellets was investigated by measuring the mass of each pellet and taking into account its geometry.

2.4.1 Vickers's hardness

The hardness measurement of the pellets was performed by using standard Vickers diamond indenter with 136° angle (Shimadzu HV2000 machine). The load applied on each pellet was 19.62 N forces (2 kg) for 20 s**.** For each sample, 10 indentations were made and the average hardness values were taken for the analysis. The indentation was measured precisely on Leica Wild M10 light microscope where two diagonals of the indentation were measured.

2.4.2 Compression tests

The compression strength test was performed on the tensile testing unit (TTU 2002) of SMITWELD 1405 testing machine. The compression strength was determined as a maximal value of the applied stress. Young's modulus of materials was also determined by compression method.

2.4.3 Fracture toughness

The linear-elastic fracture toughness of a material was determined from the stress intensity factor ΔK at which a thin crack in the material begins to grow. It is denoted as K_{Ic} and was measured in $MPa \cdot m^{1/2}$ or $ksi \cdot in^{1/2}$ units. Fracture toughness was obtained on Vickers hardness machine ZWICK, by indenting Vickers's diamond pyramid with angle 136° into HAp pellets. Depending on the behavior of the material and the appearance of the crack in the material, the crack could be half-penny shaped or elliptic shaped. Thus, the fracture toughness was determined by half penny and Palmquist model.

2.5 Biocompatibility study

2.5.1 Indirect contact assay

For indirect cytotoxicity test, 20% dimethyl sulfoxide (DMSO) and standard culture medium were used as positive and negative controls, respectively. To evaluate the short-term cytotoxicity of the material, direct and indirect contact assays were performed. Possible toxic effects of leachable released from the material were measured using the extracts of these materials. The ratio of material weight to extract fluid was 0.2 g mL^{-1} of complete culture medium with samples maintained at 37°C at 120 r/min for 72 h. The complete culture medium was serum-free 1: 1 mixture of Ham's F12 and Medium Dulbecco Modified Eagle's minimal essential medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% penicillin/streptomycin (p/s, Gibco, Grand Island, NY, USA). The supernatant was withdrawn and centrifuged to prepare the conditioned extract and filtered using 0.4 μm filters and then stored at 4°C before the cytotoxicity test. The pH value of the extraction medium was also measured.

Human fetal-osteoblast cell line, hFOB 1.19 were grown as monolayers in 1 : 1 mixture of Ham's F12 and DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 2.5 mM L-glutamine (Gibco, Grand Island, NY, USA) with 1% p/s in a humidified incubator maintained at 34°C and 5% CO_2, which was trypsinized before the experiments. Cells were seeded at a density of 50×10^3 cells/cm^2 per well (n=4) in 24-well plate using the complete culture medium incubated for 24 h. Then the medium was aspirated which was followed by the addition of 500 μL conditioned or control medium after adding 10% FBS. The inhibitive effect of the extract on the cell growth was evaluated after incubating the plate for 1, 3, and 7 days and compared with the negative control. To measure the metabolic activity of cells [3-(4,5-dimethylthiazol)-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay was applied. Metabolically active cells reduce yellow-coloured MTT to purple coloured formazan dye crystals. Briefly, 50 μL of MTT solution (5 mg mL^{-1}; Sigma-Aldrich, St Louis, MO, USA) was added to each

well and incubated for 4 h. After discarding the medium containing MTT, 350 μL DMSO was added to all wells to dissolve the formazan into a purple solution. After 10 min incubation, 100 μL aliquots from the wells were pipetted into another 96-well plate. Optical density was measured at a wavelength of 570 nm with a SPECTRA max® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The cell activity was represented as the percentage of activity expressed by cells compared to negative control. Cells in the underlying wells were labeled after 7 days of culture using the LIVE/DEAD Viability/Cytotoxicity kit (L-3224, Invitrogen Corp.), according to the manufacturer's instructions and photographed under a fluorescent microscope (Olympus, Tokyo, Japan).

2.5.2 Direct contact assay

Materials were placed in 24-well tissue culture plates and seeded with aliquots containing 50×10^3 cells/cm^2 of hFOB. Plates were incubated at 34 °C and 5% CO_2 for 7days. Cytotoxicity was measured by DNA quantification assay after 1 and 7 days of culture, whereas, the function was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) after 7 days of culture.

For DNA extraction, cell seeded materials were homogenized in lysis buffer at 56 °C followed by centrifugation of the suspension (2500 ×g, 10 min, 4 °C). The supernatant was collected and used for dsDNA analysis using NucleoSpin kit (Machery-Nagel Gmbh& Co.KG, Germany) according to the manufacturer's instructions. The concentration of extracted DNA was measured with a NanoDrop spectrophotometer ND-1000 (PeqLab, Erlangen, Germany) by photometric measurement of the optical density at 260 nm.

For PCR assay, total RNA was extracted from cells grown on the material, while cells cultured in two-dimensional monolayer were used as a control using TRIzol solution (Invitrogen) according to manufacturer's instructions, 1 ug of total RNA was used to synthesize cDNA using random primers and GoScript reverse transcriptase (Promega, Korea). 50 ng of cDNA extracted was used for PCR analysis by TProfessional standard 96 gradient machine (Biometra, Goettingen, Germany). The primer sequences are summarized in Table 1. The PCR conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide and visualized with ultraviolet transillumination (G: BOX F3, Syngene, Cambridge, UK) using a reference 100-base pair DNA ladder (GeneRuler, Fermentas, Burlington, ON, Canada). Relative gene expression was determined using image J software with GAPDH as an endogenous control.

3. Results and discussion

3.1 XRD studies

Fig. 1a and 1b show the XRD patterns of CaO and CaOH, respectively synthesized using eggshell by employing ultrasound irradiation. The XRD spectra of CaO shows peak corresponding to (111), (200), (220), (311), (222), and (400) while that of CaOH shows peak corresponding (001), (100), (011), (012), (110), (111) and (201), respectively. The XRD pattern of calcined eggshell (Fig.1a) shows peaks corresponding to calcium oxide (JCPDS-821690) as excepted. The XRD pattern of oven dried eggshells powder and treated with ammonium hydroxide formed CaOH (Fig. 1b) was found to be similar to that of CaOH and as received CaOH, exhibiting peaks corresponding to CaOH (JCPDS-841276). An earlier report of CaO on exposure to the atmosphere being converted into CaOH supports our observations[15]. Further,

the effect of sonochemical reaction on crystallinity and average crystallite size of synthesized HAp samples were obtained by X-ray diffraction analysis. The high intensity and sharp peaks in the XRD pattern in Fig. 1c-d confirm the purity and crystalline nature of EHAp and CHAp. Fig. 1c-f shows XRD patterns of HAp samples showing hkl indices (200), (201), (102), (211), (300), (220), (203), (230), and (303). The XRD results of synthesized EHAp, CHAp, EHAp600 and CHAp600 samples show increased crystallinity with sharper peaks corresponding to (JCPDS-861199) as shown in (Fig.1c-f). The lattice parameters calculated from the XRD data by least squares fit method are as listed in Table 2, which indicate that the values are comparable with those reported a-axis 9.418 Å and c-axis 6.884 Å. The broad peaks observed in (Fig.1c-f) indicate nanocrystalline nature of the synthesized powder. The average crystallite size was determined using the Scherrer's formula $D=0.9\lambda/\beta \cos\theta$, where λ is the wavelength of the incident X-rays (λ =1.54056 Å for Cu $K\alpha_1$ radiation), β is the full width at half maximum of the XRD peaks expressed in radian, θ (hkl) is the peak diffraction angle satisfying Bragg's law for the (hkl) plane and D is the crystallite size.

The particle size calculated using Scherrer's formula for all samples is shown in Table 2. The XRD results indicate that ultrasound irradiation significantly influenced the chemical interactions of the reacting species by possibly altering the rate of formation and chemical equilibrium of the calcium hydrogen phosphate phases. Another chemical species, probably a type of calcium hydrogen phosphate, may be formed in insignificant amounts.

However, in high temperature treated samples, there is less possibility of mixed phases. In HAp samples as in Fig. 1c-f, the peaks with significant intensity in the range 23–39^o and a lower intensity peak at 46–63^o were consistent with that of the $Ca_{10}(PO_4)_6(OH)_2$ phase (JCPDS No. 861199). The intensities of these peaks were noticeable to conclude the predominant existence of HAp. The particle size is related to nucleation and growth

pattern of the material and this is highly related to the degree of supersaturation in the liquid phase. In presence of ultrasound irradiation, there is the creation of micro jets and shock wave impact due to the collapse of microbubbles and these promote hot spots with very high cooling rates[36].

These effects promote chemical reactions and physical effects, thus ultrasound can be used for the synthesis of materials in liquid phase [37]. The sonication power influences the level of cavitation in the liquid and with higher power ultrasound generating the larger number of cavitation events due to more transient cavitation bubbles being formed. It can be expected that as more nucleation sites are created the particles formed around these sites are smaller for the same original reagent concentration. In addition, formed particles exposed to longer contact time with ultrasound is expected to show decreased level of agglomeration. Thus, higher ultrasound power resulted in a decrease in particle size. The results are further supported by FESEM results confirming the decrease in the level of agglomeration when ultrasound is employed in the reaction process.

3.2 FESEM studies

Scanning electron microscopy (FESEM) was used to determine the size, morphological and structural features of the synthesized nanoparticles of CaO, CaOH, EHAp, CHAp, EHAp600, and CHAp samples. Prior to the FESEM observations, small amount of powder were placed onto an FESEM specimen holder. They were then sputter coated with gold in order to minimize the charging under the electron beam. The samples were subsequently analyzed using FESEM (Hitachi-S4800, Japan) operated at 20 kV at various magnifications. Simultaneously, the elemental compositions of the samples were also investigated and confirmed using energy dispersive spectroscopy (EDS). Images in Fig. 2(a) and 2(b) depicts the FESEM images obtained from CaO and CaOH,

respectively prepared using eggshell. The images show agglomeration of fine spherical particles of CaO and CaOH. Similarly, Fig. 2(c) and 2(d) shows FESEM images of EHAp (as-prepared) and EHAp600 (calcined at 600 ^{o}C) obtained from eggshell employing ultrasound irradiation. On the other hand, FESEM images obtained from CHAp (as-prepared) and CHAp600 (calcined at 600 ^{o}C) prepared using chemical synthesis employing ultrasound irradiation are shown in Fig. 2(e) and 2(f), respectively. These images show spherical nanoparticles of HAp with relatively less agglomeration. Details of the observed average crystal size from FESEM images are tabulated in Table 2. These are similar to the particle morphologies reported elsewhere[38,39]. The FESEM results compare favorably with the calculated grain size from the XRD spectra. To further confirm the elemental composition of the as-prepared and calcined EHAp, EHAp600, CHAp, and CHAp600 samples, the EDX spectra were recorded as shown in Fig. 2g. Peaks corresponding to Ca, P, and O characteristic of HAp were observed in addition to traces of Mg and C impurities originating from precursors.

3.3 Dynamic light scattering (DLS) analysis

Dynamic light scattering analysis was performed to determine the size distribution of the eggshell after crushing, EHAp, EHAp600, CHAp, and CHAp600 nanoparticles and the results are shown in Fig. 3. The samples were dispersed in distilled water and sonicated for 10 min to minimize agglomeration which is a common phenomenon. DLS conducted after eggshell after crushing, EHAp, EHAp600, CHAp, and CHAp600 nanoparticles show the average diameter of 160 nm, 300 nm, 320 nm, 310 nm and 390 nm, respectively. There is a large variation in particle size distribution and it is attributed to agglomeration of the particles and moreover, the shape of the agglomerated particles (large grains) may be irregular and not spherical. Furthermore, the size and size distribution measurement in DLS does not represent exact size as it the hydrodynamic

size of the particles or grains. The average size of the particles could be elucidated from XRD measurements and the data is summarized in Table 2. The size of EHAp and CHAp increased after calcined at 600 C temperature, which may be due to the agglomeration of particles at high temperature. This significant change in the size and crystallinity is likely to play a major role in the mechanical properties such as hardness, fracture toughness, and compression strength.

3.4 FT-IR studies

The FT-IR (CARRY 600 Series, Japan) spectra were obtained over the region 400–4,000 cm^{-1} using attenuated total reflectance (ATR) technique with a spectral resolution of 4 cm^{-1}. The ATR unit permits the spectra collection without any specific sample preparation. Fig. 4a-f shows the FTIR spectra of CaO, CaOH, EHAp, EHAp600, CHAp, and CHAp600, respectively and found to be in agreement with earlier reports. The IR spectra of all the samples show broad bands in the high energy region that are probably water related bands. The sharp band at 3567 cm^{-1} in the HAp spectrum is assigned to the OH stretching mode. The PO_4^- derived bands appear at 570, 609, 966 and 1030–1093 cm^{-1}. The bands at 570 and 609 cm^{-1} are from the ν_4 vibrations of the O–P–O mode. The strong bands at 966 and 1030-1093 cm^{-1} corresponds to PO_4^- functional group resulting from the ν_1 P–O symmetric stretching vibrations [28,38,40]. The CO_3^- derived bands are observed at 870 and 1402–1460 cm^{-1} [9,28,41]. It might be due to the adsorption of atmospheric carbon dioxide during the sample preparation. However, from the XRD, it was found that the CO_3 groups did not influence the purity of HAp. There are two shoulder peaks at 630 and 3570 cm^{-1}, which are typical stretching vibration and bending modes of HAp. The bands at 1635 and 3399-3471 cm^{-1} reflecting H_2O are also observed [9,28,40]. The weak peak at 1644 cm^{-1} corresponds to the CO_3^- group. The band at 3432 cm^-

[1] corresponds to absorbed water, while the weak peak at 3570 cm^{-1} corresponds to the vibrations of OH^- ions in the HAp lattice [9,25,42]. This indicates that the samples are of good quality. Moreover, the corresponding IR bands are given in Table 3.

3.5 Raman studies

The samples were analyzed by Raman spectroscopy using a Raman spectrometer (STR-150 series, Japan). The excitation source was a laser with an excitation wavelength of 532 nm. The samples were placed on a glass slide prior to the analysis and the laser beam was focused on the sample surface for acquiring the spectra. The Raman spectra could give only limited information about the properties of the Raman tensors because of the randomly oriented microcrystallites in the powder samples. Fig. 5a-f shows the Raman spectra of CaO, CaOH, EHAp, EHAp600, CHAp, and CHAp600, respectively. The Raman spectra of EHAp, EHAp600, CHAp and CHAp600 show sharp bands indicating a high degree of crystallinity. The strongest Raman active ν_1 PO_4 mode (Fig. 5c-f) appears in the spectra of the HAp samples at 961 cm^{-1} and ν_2 PO_4 mode shows peaks at 429 cm^{-1} the spectra of the well-crystallized samples. It can be observed that the spectrum is characterized by a very strong peak at 961 cm^{-1} and a peak at 429 cm^{-1} which are the characteristics vibrations of HAp. Thus, using this information the existence of HAp could be ensured.

3.6 Mechanical properties study

The density or more precisely the volumetric mass density of a substance is its mass per unit volume. The averaged results of 20 pellets of each material are shown in Fig. 6a. The density varied from 0.48 to 0.52 g/cm^3 because the same force was used for preparing the pellet using all materials under consideration. Density is observed to be slightly higher in the case of EHAp and CHAp600 materials. The Vickers hardness measurements revealed that the hardness is higher in EHAp material when compared to

EHAp600 because the process of sintering at a higher temperature at 600 °C before the pellets preparation might have influenced the crystal microstructure. On the other hand, in the case of CHAp and CHAp600, the difference in hardness is smaller owing to the agglomeration of the crystals due to the heating at 600 °C. This increase in grain size in the powders decrease the hardness in the case of CHAp600 pellets. The results of hardness measurements are shown in Fig. 6b.

Fracture toughness was measured by Vickers indentation method. Evaluating of the fracture toughness depends on the shape of the cracks (Palmquist or Half penny-shaped) [43]. Fracture toughness is the ability of a material with the crack to resist fracture. It is most important properties of the material for designing applications.

The linear-elastic fracture toughness of a material is determined from the stress intensity factor ΔK at which a thin crack in the material begins to grow. It is denoted as K_{Ic} and is measured in MPa·m$^{1/2}$ or ksi·in$^{1/2}$ units. Depending on the behavior of the material and the crack appearance in the material, the crack can be Half-penny shaped or elliptic shaped. Accordingly, in literature, there exist two basic models like Half-penny shaped model and Palmquist model. If the crack is short and shallow ($\frac{c}{a} \leq 2$) Palmquist model is used for evaluating of the fracture toughness by curve fitting, otherwise Half-penny model is used when crack is long and deep ($\frac{c}{a} \geq 2$).

Many different equations exist in literature which helps to evaluate the fracture toughness of material by Vickers hardness indenting technique.

Niihara and co-authors [44] suggested using following equation for Palmquist model when ($\frac{c}{a} \leq 2$).

$$K_{IC} = 0.0089 \left(\frac{E}{H_v}\right)^{\frac{2}{5}} \frac{P}{ac^{1/2}} \qquad \text{(eq. 1)}$$

Lawn and co-authors [45] revealed following formula for the longer cracks which behave according to Half-penny model.

$$K_{IC} = 0.014\left(\frac{E}{H_v}\right)^{1/2}\frac{P}{c^{2/3}} \qquad \text{(eq. 2)}$$

At the same time, Anstis and co-author suggested equation for Half-penny model when geometry of the crack is according to $\frac{c}{a} \geq 2$.

$$K_{IC} = 0.016\left(\frac{E}{H_v}\right)^{1/2}\frac{P}{c^{2/3}} \qquad \text{(eq. 3)}$$

In this study, all the three equations are used for the determination of fracture toughness of CHAp, CHAp600, EHAp and EHAp600. Fig. 6c represents the results of fracture toughness K_{Ic} study and designated as a and b, which means that the cracks are shorter (Palmquist cracks) and could be respected by equation 1 used for fracture toughness determination. Designation c means that the cracks are bigger and half penny-shaped and thus for the fracture toughness determination equation 3 is used. CHAp material shows the highest ability of a material with the crack to resist fracture. Further the results of compression strength are presented in Figure 6d. The highest compression strength is found in the case of EHAp material. The compression strength of the other three materials was almost the same. Figure 6e shows the results of elastic modulus with the maximum elastic modulus is measured in EHAp material and the least in the case of CHAp material, which pointed on higher elastic deformability of CHAp material. The artificial bone material should be stiff enough, flexible and able to resist deformation to making loading possible similar to original bone. However, the bone is a complex structure made up of organic (collagen) and inorganic (hydroxyapatite) material, thus the strength of the bone is characterized by its material composition and structure. A

characteristic feature of a bone is that it possesses stiffness, flexibility, lightness and strength. Thus, these properties should be achieved while designing the material for bone regeneration application. The compression strength is up to 230 MPa in cortical bone tissue, while in EHAp (Fig. 6e) it is found to be about 55 MPa. This is higher compared to the other samples and mostly alike cancellous bone and some of the cortical bone tissues. The fracture toughness is 2-12 MPa $m^{1/2}$ in the case of cortical bone, while Fig. 6c shows the maximum in case of CHAp and minimum in case of EHAp samples still suitable for cortical bone. The Young's modulus ranges from 0.5 to 30 GPa in cancellous and cortical bone tissues. Figure 6e depicts that the EHAp is having Young's modulus of around 4 GPa which is highest among the other samples and thus all the samples are suitable for both the bone tissues.

The pellets were prepared by uniaxial compression at ambient temperature under high pressure about 15000 N (763 MPa) load applied. The pellets were tested according to ASTM C1424-15 (Standard Test Method for Monotonic Compressive Strength of Advanced Ceramics at Ambient Temperature) and the data is shown in Fig. 7. Although pellets were prepared under quite high pressure, still there is the possibility of the presence of porosity which was measured by taking cross-section micrograph of pellets by SEM. It is difficult to get a precise measure of porosity, however, we have tried to measure the porosity of EHAp and CHAp samples considering their density and by measuring the percentage of porosity area in different SEM cross-section micrographs by taking into account several micrographs with five different pellets of EHAp and CHAp each. The pellets were cut vertically and cross-section area SEM micrographs were taken in different places. The porosity was determined by measuring the cross-section of SEM images by cutting pellet (by using the AutoCAD software). The total

porous area measured with respect to the total area of the micrograph. Percentage of porosity was calculated by the following equation.

Porosity (%) = (Porous area in micrograph /Total area of micrograph) X 100 (eq. 4)

Porosity found in EHAp sample is about 4% and in CHAp 0.5% (Fig. 7). The influence of the relative porosity on mechanical properties of porous ceramic varies in an exponential way i.e. increase in porosity decrease the properties usually described by mathematical relations: power-law or exponential dependence with porosity[46,47]. The porosity could affect the fracture toughness and other mechanical properties but for evaluation, this technique/equations is used to predict and compare different conditions.

The fracture toughness (K_{Ic}) of pellets was determined (Fig. 8) by the indentation of the Vickers diamond pyramid with the angle 136° at higher loads to cause the crack which appeared at each corner of the indentation. Bigger cracks mean lower fracture toughness and the smaller cracks mean higher toughness. For measuring fracture toughness it is important to choose the appropriate model for calculation. In the case of bigger cracks, two cracks on each opposite corner of impression (observed from the top) are connected in one crack. In this case, Halfpenny model to be used. However, in smaller cracks, two cracks exists in opposite corner in Vickers impression. These cracks are shallow cracks, thus in this situation Palmquist model to be used[43]. The criteria to decide the choice of model is defined by c/a ratio. Both diagonals (2·a1 and 2·a2) of the indentation and each of the crack lengths (l1, l2, l3 and l4) were measured after testing by using the Palmquist (c/a is ≤ 2.1) versus Half Penny (c/a is > 2.1) model, and the fracture toughness was evaluated. Fracture toughness defines the behavior of material that contains a defect, independent of its geometry. Detail study of using different

models and equations for calculation of fracture toughness have been done on different materials by authors[43]. Most suitable equation proposed by authors for fracture toughness calculations in case of our materials are given in Fig. 8.

The porosity influences the material properties. Although this method for measuring of fracture toughness for ceramic powder material may not be appropriate to obtain a precise and exact result, still it is suitable for brittle material to evaluate with less quantity to predict and compare the mechanical properties. However, this study is concerned with the comparison of similar ceramic material obtain by different sources. Thus, during the evaluation of fracture toughness, there may be small error exists but the same magnitude for all investigated materials because of similar kind of materials. In order to obtain comparative study, we believe that this approach is appropriate. The fracture toughness is found to be higher in CHAp than EHAp, the reason may be that less porosity is found in CHAp than EHAp and also SEM micrograph and particle size distribution showed smaller particle size in CHAp than EHAp. However, the other mechanical properties obtained are superior in case of EHAp than CHAp although the EHAp is comparatively porous than CHAp. It is may be due to the better crystallization and more uniformity in particle size distribution in the case of EHAp. The preparation of EHAp and CHAp is done by similar method and conditions but calcium precursors are from different sources which might have some influence on the mechanical properties.

Hydroxyapatite (HAp) is a promising implant material and its use in load-bearing applications such as artificial joints have been constrained by the low toughness (0.8–1.2 MPa $m^{1/2}$) and low flexural strength (<140 MPa) of the ceramic body. The enhancement of the mechanical properties of HAp is usually attained by compaction and sintering at high temperature or heat treatment. Uni-axial pressing is the common

approach of achieving compaction but the sintered body tends to lose its uniformity and develop cracks. Sintering of HAp at elevated temperatures often leads to change in HAp phase and it affects the mechanical properties. Moreover, sintering at elevated temperatures has the tendency to eliminate the functional group OH in the HAp matrix and this would result in the decomposition of HAp phase to form α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP)[1]. Thus appropriate heat treatment is essential. The mechanical properties of sintered hydroxyapatite (HAp) at various sintering temperatures in some reported studies and in comparison with our study is given in Table 4.

3.7 Biocompatibility and cell study

3.7.1 Indirect contact assay

Phase contrast microscopy (Fig. 9 (a-d)) and MTT assay (Fig. 10) show that the negative control displayed no effect on cell growth and morphology, while the toxic effect of DMSO was clear; cells displayed severe morphological changes and they were not able to proliferate. CHAp and EHAp materials displayed no inhibitive effect on cell viability or growth compared to the negative control. Only a few cells were positive for EthD-1 staining as shown in Fig. 9 (e-h); indicating that the viability was maintained over time upon the culture of cells. EHAp samples revealed a relatively higher number of dead cells compared to CHAp and negative control group, which might be caused by leaching of toxic materials or the lower pH (8.27) compared to pH of 8.49 and 8.49 for CHAp and negative control samples, respectively. This data suggests that EHAp and CHAp do not inhibit the normal growth of osteoblast and that their use as orthopaedic implants would not affect the normal physiological microenvironment after in vivo implantation.

3.7.2 Direct contact assay

DNA extraction of the cells grown on the materials (Fig. 11) shows their ability to attach and proliferate on the material surfaces. DNA concentrations show an increase in cell number from day 1 to day 7. These concentrations were non-significant compared to that of negative control (658 ± 17.6 ng in CHAP, 636.7 ± 29.1 ng in EHAp, and 687.6 ± 30.36 ng in negative control). During the bone formation process, osteoblasts produce a variety of proteins such as osteocalcin (OC), osteopontin (OP), osteonectin (ON), collagen 1 (COL-1) and bone morphogenetic protein-2 (BMP-2). The expressions of mRNA markers of these proteins were investigated by RT-PCR analysis and normalized using GAPDH (Fig. 12) to compare the osteogenic potentials of hFOB grown on the scaffolds. A densitometric analysis of the mRNA expression of cDNA prepared from cells cultured on CHAp samples shows a significant up-regulation in OC, and BMP-2 compared to EHAp and negative control samples (OC, 1.9 ± 0.13 fold; BMP-2, 2.6 ± 0.95 fold for CHAp samples and OC, 1.08 ± 0.11 fold; BMP-2, 0.95 ± 0.21 fold). This suggests that CHAp has a noticeable role in the osteogenic activity of osteoblasts

OC and OP are osteoblast-specific marker genes [22]. OC is a major non-collagenous protein component of the bone extracellular matrix (ECM) which considered as a relatively late marker of osteoblast differentiation related to mineralization produced by both osteoblasts and osteoclasts. OP is normally found in specific regions of bone in vivo such as cement lines in remodeling bone and is involved in bone remodeling, cell adhesion and ECM mineralization [48]. In vitro, osteopontin is commonly associated with the formation of a collagen-free cement layer on which bone is subsequently deposited. Collagen-1 aids in the deposition of collagen, the most abundant ECM protein in bone. BMP-2 has been demonstrated to play a critical role in accelerating osteoblast differentiation and bone formation during embryonic skeletal development and postnatal bone remodeling. In the present study, the up-regulated expression of bone-related markers may indicate the more osteopromotive activity of CHAp.

4. Conclusion

HAp nanoparticles were synthesized by means of ultrasonication method, using eggshells waste as a calcium source and ammonium dihydrogen phosphate as reactants. Hydroxyapatite is a ceramic that is generally considered to be a viable substitute for bone material in many clinical biomedical and tissue engineering applications. In this study, the influence of ultrasound on the synthesis of nano-HAp powders was examined. Nano-HAp particles in the nanometer size range and spherical morphology were produced using the ultrasound-mediated chemical technique. It is found that the crystalline structure and morphology of the resulting nano-HAp powders was influenced by ultrasonication. It was evident that the presence of ultrasound in the synthesis process promoted the chemical reactions and physical effects that subsequently produced the ultrafine nano-HAp particles. Ultrasound-assisted synthesis process produced nano-HAp powders with the same crystalline structure and morphology. For an orthopaedic application, the mechanical strength, fracture toughness, and elastic modulus are important properties in terms of resisting the load. In order to achieve the balanced strength and toughness the EHAp material found to be most appropriate as the results obtained in the case of compression strength, elastic modulus, and fracture toughness. The in vitro bioactivity and biocompatibility study of EHAp and CHAp material showed that the scaffolds facilitate the cell attachment and proliferation. hFOB cells cultured on the scaffolds, and the results showed EHAp and CHAp scaffold highly promote the bioactivity, osteoconductivity and cell differentiation. According to the data presented in this study, we can conclude that EHAp and CHAp did not affect the shape and the growth of cells, indicating their biocompatibility and suggesting that their application would be beneficial as implants for bone regeneration.

Acknowledgements

V.H.I. is thankful to UGC, New Delhi, for a research fellowship (File No. RGNF-F1-17.1/2012-13/RGNF-2012-13-SC-MAH-35362/ [SA-III/website]). A.A.K is thankful to CSIR, New Delhi, for the research fellowship (File no.09/809(0013)/2012-EMR-I). The authors are thankful to RGSTC, Mumbai, UGC-DAE-CSR, Indore (Project Ref. No: CSR-I/CRS-48/48) and UGC, New Delhi (File No. 41-370/2012 (SR)) for the financial support. JYC is thankful to the Ministry of Science and Technology, R.O.C. contract no. MOST 105-2119-M-011-002.

References

1. Poinern GEJ, Brundavanam RK, Fawcett D. Nanometre Scale Hydroxyapatite Ceramics for Bone Tissue Engineering. Amer. J. Biom. Engin. 2013;**3**(6):148-168.
2. Ngiam M, Nguyen LT, Liao S, Chan CK, Ramakrishna S. Biomimetic nanostructured materials: Potential regulators for osteogenesis. Ann Acad Med Singapore 2011;40(5):213-22.
3. Ozdemir T, M. Higgins A, L. Brown J. Osteoinductive Biomaterial Geometries for Bone Regenerative Engineering. Curr. Pharm. Des. 2013;**19**(19):3446-3455.
4. Krishna DSR, Siddharthan A, Seshadri SK, Kumar TSS. A novel route for synthesis of nanocrystalline hydroxyapatite from eggshell waste. J. Mater. Sci. - Mater. Med. 2007;**18**(9):1735-1743.

5. Rivera-Muñoz EM. Hydroxyapatite-Based Materials: Synthesis and Characterization. Biomedical Engineering-Frontiers and Challenges 2011:978-953.
6. Jiang H, Zuo Y, Zou Q, Wang H, Du J, Li Y, Yang X. Biomimetic Spiral-Cylindrical Scaffold Based on Hybrid Chitosan/Cellulose/Nano-Hydroxyapatite Membrane for Bone Regeneration. ACS Appl. Mater. Interfaces 2013;**5**(22):12036-12044.
7. Zhou H, Lee J. Nanoscale hydroxyapatite particles for bone tissue engineering. Acta Biomater. 2011;**7**(7):2769-2781.
8. Kim T-G, Park B. Synthesis and growth mechanisms of one-dimensional strontium hydroxyapatite nanostructures. Inorganic chemistry 2005;44(26):9895-9901.
9. Wang Y, Chen J, Wei K, Zhang S, Wang X. Surfactant-assisted synthesis of hydroxyapatite particles. Mater. Lett. 2006;60(27):3227-3231.
10. He QJ, Huang ZL. Controlled growth and kinetics of porous hydroxyapatite spheres by a template-directed method. J. Cryst. Growth 2007;300(2):460-466.
11. Rivera EM, Araiza M, Brostow W, Castano VM, Dıaz-Estrada JR, Hernandez R, Rodrıguez JR. Synthesis of hydroxyapatite from eggshells. Mater. Lett. 1999;**41**(3):128-134.
12. Acevedo-Dávila JL, López-Cuevas J, Vargas-Gutierrez G, Rendón-Angeles JC, Méndez-Nonell J. Chemical synthesis of bone-like carbonate hydroxyapatite from hen eggshells and its characterization. Bol. Soc. Esp. Ceram. Vidrio 2007;46(5):225-231.
13. Goller G, Oktar FN, Ozyegin LS, Kayali ES, Demirkesen E. Plasma-sprayed human bone-derived hydroxyapatite coatings: effective and reliable. Mater. Lett. 2004;58(21):2599-2604.
14. Akram M, Ahmed R, Shakir I, Ibrahim WAW, Hussain R. Extracting hydroxyapatite and its precursors from natural resources. Journal of Materials Science 2014;49(4):1461-1475.

15. Siva Rama Krishna D, Siddharthan A, Seshadri SK, Sampath Kumar TS. A novel route for synthesis of nanocrystalline hydroxyapatite from eggshell waste. Journal of Materials Science: Materials in Medicine 2007;18(9):1735-1743.
16. Xu J, Khor KA, Dong Z, Gu YW, Kumar R, Cheang P. Preparation and characterization of nano-sized hydroxyapatite powders produced in a radio frequency (rf) thermal plasma. Mater. Sci. Eng., A 2004;334:101-108.
17. Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R. Enhanced osteoclast-like cell functions on nanophase ceramics. Biomaterials 2001;**22**(11):1327-1333.
18. Fujihara K, Kotaki M, Ramakrishna S. Guided bone regeneration membrane made of polycaprolactone/calcium carbonate composite nano-fibers. Biomaterials 2005;26(19):4139-4147.
19. Ngiam M, Liao S, Patil AJ, Cheng Z, Yang F, Gubler MJ, Ramakrishna S, Chan CK. Fabrication of mineralized polymeric nanofibrous composites for bone graft materials. Tissue Eng. Part A 2008;15(3):535-546.
20. Rao RR, Roopa HN, Kannan TS. Solid state synthesis and thermal stability of HAP and HAP–β-TCP composite ceramic powders. J. Mater. Sci. Mater. Med. 1997;8(8):511-518.
21. Pramanik S, Agarwal AK, Rai KN, Garg A. Development of high strength hydroxyapatite by solid-state-sintering process. Ceram. Int. 2007;33(3):419-426.
22. Ingole VH, Hussein KH, Kashale AA, Gattu KP, Dhanayat SS, Vinchurkar A, Chang J-Y, Ghule AV. Invitro Bioactivity and Osteogenic Activity Study of Solid State Synthesized Nano-Hydroxyapatite using Recycled Eggshell Bio–waste. ChemistrySelect 2016;1(13):3901-3908.
23. Bezzi G, Celotti G, Landi E, La Torretta TMG, Sopyan I, Tampieri A. A novel sol–gel technique for hydroxyapatite preparation. Mater. Chem. Phys. 2003;**78**(3):816-824.

24. Weng W, Han G, Du P, Shen G. The effect of citric acid addition on the formation of sol–gel derived hydroxyapatite. Mater. Chem. Phys. 2002;74(1):92-97.
25. Wang Y, Zhang S, Wei K, Zhao N, Chen J, Wang X. Hydrothermal synthesis of hydroxyapatite nanopowders using cationic surfactant as a template. Mater. Lett. 2006;60(12):1484-1487.
26. Prélot B, Zemb T. Calcium phosphate precipitation in catanionic templates. Mater. Sci. Eng., C 2005;25(5):553-559.
27. Ioku K, Yamauchi S, Fujimori H, Goto S, Yoshimura M. Hydrothermal preparation of fibrous apatite and apatite sheet. Solid State Ionics 2002;151(1):147-150.
28. Liu J, Ye X, Wang H, Zhu M, Wang B, Yan H. The influence of pH and temperature on the morphology of hydroxyapatite synthesized by hydrothermal method. Ceram. Int. 2003;29(6):629-633.
29. Lim GK, Wang J, Ng SC, Chew CH, Gan LM. Processing of hydroxyapatite via microemulsion and emulsion routes. Biomaterials 1997;18(21):1433-1439.
30. Sun Y, Guo G, Tao D, Wang Z. Reverse microemulsion-directed synthesis of hydroxyapatite nanoparticles under hydrothermal conditions. J. Phys. Chem. Solids 2007;68(3):373-377.
31. Lim GK, Wang J, Ng SC, Gan LM. Formation of nanocrystalline hydroxyapatite in nonionic surfactant emulsions. Langmuir 1999;15(22):7472-7477.
32. Cao L-y, Zhang C-b, Huang J-f. Synthesis of hydroxyapatite nanoparticles in ultrasonic precipitation. Ceram. Int. 2005;31(8):1041-1044.
33. Li-yun C, Chuan-bo Z, Jian-feng H. Influence of temperature,[Ca< sup> 2+</sup>], Ca/P ratio and ultrasonic power on the crystallinity and morphology of hydroxyapatite nanoparticles prepared with a novel ultrasonic precipitation method. Mater. Lett. 2005;59(14):1902-1906.

34. Qiu Y, Xia H, Jiang H. Fabrication of nano-hydroxyapatite using a novel ultrasonic atomization precipitation method. J. Nanosci. Nanotechnol. 2010;10(3):2213-2218.
35. Zhijie H. Preparation of Hydroxyapatite Nanometer-size Needle-like Crystal by Utrasonic Treatment. Mate. Rev. 2003:S1.
36. Beckett EL, Lawrence NS, Tsai YC, Davis J, Compton RG. Bioanalytical utility of sonovoltammetry. J. Pharm. Biomed. Anal. 2001;26(5–6):995-1001.
37. Perusich SA, Alkire RC. Ultrasonically Induced Cavitation Studies of Electrochemical Passivity and Transport Mechanisms: II . Experimental. J. Electrochem. Soc. 1991;138(3):708-713.
38. Poinern GE, Brundavanam RK, Mondinos N, Jiang Z-T. Synthesis and characterisation of nanohydroxyapatite using an ultrasound assisted method. Ultrason. Sonochem. 2009;**16**(4):469-474.
39. Zhou Z-H, Zhou P-L, Yang S-P, Yu X-B, Yang L-Z. Controllable synthesis of hydroxyapatite nanocrystals via a dendrimer-assisted hydrothermal process. Mater. Res. Bull. 2007;42(9):1611-1618.
40. Kannan S, Lemos AF, Ferreira JMF. Synthesis and mechanical performance of biological-like hydroxyapatites. Chem. Mater. 2006;**18**(8):2181-2186.
41. Gómez Morales J, Rodríguez Clemente R, Armas B, Combescure C, Berjoan R, Cubo J, Martinez E, García Carmona J, Garelik S, Murtra J. Controlled nucleation and growth of thin hydroxyapatite layers on titanium implants by using induction heating technique. Langmuir 2004;**20**(13):5174-5178.
42. Panda RN, Hsieh MF, Chung RJ, Chin TS. FTIR, XRD, SEM and solid state NMR investigations of carbonate-containing hydroxyapatite nano-particles synthesized by hydroxide-gel technique. J. Phys. Chem. Solids 2003;64(2):193-199.

43. Moradkhani A, Baharvandi H, Tajdari M, Latifi H, Martikainen J. Determination of fracture toughness using the area of micro-crack tracks left in brittle materials by Vickers indentation test. J. Adv. Ceram. 2013;2(1):87-102.
44. Niihara K, Morena R, Hasselman DPH. Evaluation ofKIc of brittle solids by the indentation method with low crack-to-indent ratios. Journal of Materials Science Letters 1982;1(1):13-16.
45. Chen J, Bull SJ. Indentation fracture and toughness assessment for thin optical coatings on glass. Journal of Physics D: Applied Physics 2007;40(18):5401.
46. Le Huec JC, Schaeverbeke T, Clement D, Faber J, Le Rebeller A. Influence of porosity on the mechanical resistance of hydroxyapatite ceramics under compressive stress. Biomaterials 1995;16(2):113-118.
47. Liu D-M. Influence of porosity and pore size on the compressive strength of porous hydroxyapatite ceramic. Ceram. Int. 1997;23(2):135-139.
48. Salgado A, Coutinho O, Reis R. Novel starch-based scaffolds for bone tissue engineering: cytotoxicity, cell culture, and protein expression. Tissue engineering 2004;10(3-4):465-474.
49. Muralithran G, Ramesh S. The effects of sintering temperature on the properties of hydroxyapatite. Ceram. Int. 2000;26(2):221-230.
50. Ramesh S, Tan CY, Tolouei R, Amiriyan M, Purbolaksono J, Sopyan I, Teng WD. Sintering behavior of hydroxyapatite prepared from different routes. Materials & Design 2012;34:148-154.
51. Prokopiev O, Sevostianov I. Dependence of the mechanical properties of sintered hydroxyapatite on the sintering temperature. Mater. Sci. Eng., A 2006;431(1–2):218-227.

Figure Captions

Figure 1: Representative XRD spectra of (a) CaO and (b) CaOH (CaO obtained from eggshell treated with ammonium hydroxide.), (c) EHAp and (d) EHAp600 are as-prepared and 600 ^{o}C calcined HAp, respectively, obtained from eggshell employing ultrasound irradiation and (e) CHAp and (f) CHAp600 are as-prepared and 600 ^{o}C calcined HAp, respectively, obtained using chemical synthesis employing ultrasound irradiation

Figure 2: Representative FESEM images of (a) CaO, (b) CaOH, (c) EHAp, (d) EHAp600, (e) CHAp, and (f) CHAp600 and (g) respective EDX spectra of HAps.

Figure 3: Representative DLS spectra of (a) Eggshell, (b) EHAp, (c) EHAp600, (d) CHAp, and (e) CHAp600.

Figure 4: Representative ATR-FTIR spectra of (a) CaO, (b) CaOH, (c) EHAp, (d) EHAp600, (e) CHAp, and (e) CHAp600

Figure 5: Representative Raman spectra of (a) CaO, (b) CaOH, (c) EHAp, (d) EHAp600, (e) CHAp (f) CHAp600

Figure 6: Comparative results of the mechanical properties of EHAp, EHAp600, CHAp, and CHAp600 (a) density of pellets, (b) hardness, (c) fracture toughness, (d) compression strength and (e) elastic modulus.

Figure 7 : Porosity measurement of the pellets.

Figure 8 : Model for fracture toughness measurements.

Figure 9: (a-d) Representative phase contrast images of human fetal-osteoblast cells at day 7 after incubation with (a) standard culture media as a negative control, (b) extract of CHAp samples, (c) extracts of EHAp samples and (d) culture media containing 20% DMSO as a positive control. (e-h) LIVE/DEAD staining images; the green dye calcein AM exclusively stains viable cells. Whereas the dead cells with compromised plasma membrane integrity are stained by the red dye ethidium homodimer. (Scale bar; (a-d) = 200 µm, (e-h) = 100 µm)

Figure 10: MTT assay for human fetal-osteoblast cells cultured with the negative control, CHAp, EHAp, and positive control extracts at various incubation periods of 1, 3 and 7 days. Results are based on the optical density measurements and were normalized to the negative control. Error bars represents mean ± standard deviation for $n = 4$.

Figure 11: DNA quantification of the cells cultured on CHAp and EHAp samples for 1 and 7 days were used as an indicator for cell proliferation on the material surfaces (n=4).

Figure: 12 (a) Gel electrophoresis of the PCR products on 1.5% agarose and measurement of the bands density using Image J software while GAPDH was used as endogenous control (b) Error bars represent mean ± standard deviation (n=4).