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Bone precursor from biowaste of Albaha city of Saudi Arabia: Synthesis, characterization and *in vitro* biocompatibility

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Abstract
At present hydroxyapatite is being frequently used for diverse biomedical purposes as it possesses excellent biocompatibility, osteoconductivity and non-immunogenic characteristics. The aim of present work was to design novel zero-dimensional (0D) biomimetic hydroxyapatite nanoparticles as bone extracellular matrix. For this reason we for the first time utilized bio-waste of cow bones of Albaha city. The obtained bone bio-waste was consumed for extraction of natural bone precursor hydroxyapatite. A very straightforward approach has been used to synthesize hydroxyapatite nanoparticles through calcinations of local cow bones without further supplementation of chemicals/or compounds. The obtained hydroxyapatite powder was ascertained using physicochemical techniques such as XRD, SEM, FTIR and EDX. These analyses clearly show that hydroxyapatite from native cow biowaste is biologically and physico-chemically comparable to standard hydroxyapatite, commonly used for biomedical purposes. The cell viability and proliferation over the prepared hydroxyapatite was confirmed with CCk-8 colorimetric assay. The morphology of the cells growing over the nano-hydroxyapatite shows that natural hydroxyapatite promotes cellular attachment and proliferation. Hence, the as-prepared nano-hydroxyapatite can be considered as economical source of bone precursor hydroxyapatite for bone tissue engineering. Taking into account the projected demand for reliable bone implants, the present research work formulate to use environment friendly methods to convert bio-waste of Albaha city into novel nano-hydroxyapatite scaffolds. Besides, being an initial step towards accomplishment of projected demands of bone implants in Saudi Arabia, our study will also help in reducing the environmental burden by recycling of bio-wastes of Albaha city.
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Keywords: Albaha city, Saudi Arabia, Implants, Hydroxyapatite, Native Cow, Bio-waste

Introduction

Tissue engineering scaffolds which operate as an extracellular matrix (ECM) to interact to living cells seems to be an excellent platform for regeneration and fixation of bone defects. It has been widely accepted that chemical composition and physical structural properties of scaffolds are matter of great concerns in fabricating ultimate scaffolds for tissue regeneration appliances [26]. Hydroxyapatite [HAP, $Ca_{10}(PO_4)_6(OH)_2$] has been recognized as the vital constituent of inorganic composition of bone in human beings. HAP has attracted attention to a great extent due of its biocompatibility and its rank as main inorganic component in teeth and bones [6]. Werner in 1786 initially introduced the term apatite [22]. HAP is a classic biomineral that is plentiful in higher organisms. For this reason it can be used as bone scaffolds [22] and luminescence materials [29]. Besides, it also possess loads of essential applications in drug delivery, [11, 17] and biomedical engineering [25] owing its chemical and biological likeness with the mineral composition of human bones and teeth [6, 9, 20].

Inspired by intrinsic properties of HAP, the present research proposes to make use of unexplored bio-waste of discarded bones of native cows of Albaha city. The bone bio-waste has been explored for the extraction of natural bone precursor HAP. Up to now, a variety of HAP derivatives, for example; carbonated HA [7, 8, 27], strontium HA [23, 28], F-substituted HA [12] and HA-based nanocomposites [15] have been reported. Previous workers have utilized a number of approaches such as emulsion [21], hydrothermal [19], solvothermal technique [18], sol-gel [4], biomimetic process [13] and microwave irradiation [16] for synthesis of HAP. In the present study, we utilized a facile hydrothermal approach for synthesis of biologically preferable HAP. Extraction of HAP from

Albaha bio-wastes is biologically safe (as no foreign chemicals are required) and economically desirable process as the projected demand of HAP bio-ceramics has been increased drastically in recent years throughout the world [2]. However, till now no study has been undertaken for the conversion of biowaste of local cow bones into bone precursor HAP. The natural HAP possesses a great potential for preparation of bone scaffolds. Given to understand the importance and need of efficient and cheap bone implants, the main aim of this research was to design cost effective strategy for the synthesis of biomimetic nanotextured HAP using cow bone wastes collected from Albaha city. HAP was extracted bio-chemically from disposed of femur bones and their potential for biomedical application was studied.

Materials and Methods

Extraction of natural bone precursor HAP from biowaste of cow bones of Baha city of KSA

To sum up the practical procedure, the discarded cow femur bones were collected from the Albaha city of Saudi Arabia and have been cut down into small pieces. The pieces of bones were washed with care with water and acetone to remove fats, connective tissue and other contaminants. After removal of adulterants, the bones were again washed with distilled water 2-3 times. Then, the bones were dried at 160 °C for 2 days. The cleaned dried bones were then grind to small particle which were further grinded by ball milling to decrease the particle size.

The natural HAP was extracted from the cleaned bones following Barakat *et al* procedure [2, 3]. All the chemicals were of analytical grade and used as received without further purification. All experiments were conducted under ambient conditions. Briefly, the left over bones of Albaha cows were used for extraction of very worthy and valuable natural bone precursor HAP. In a typical process, clean bones were kept in an open alumina crucible and heated in the furnace (Lenton Thermal Designs Ltd., South Korea). The treated bones were subjected to heat treatment, (calcined at 850 °C at heating rate of 10 °C/min) for 2 hours to produce natural HAP ceramics. Afterwards; the acquired ceramics were crushed into powder to get particle size dimensions.

Physico-chemical categorization

In order to determine the crystalline phase of as-synthesized HAP ceramics, X-ray diffraction analysis was carried out. The XRD analysis of as-synthesized cow bone HAP was recorded on a Rigaku/Max-3A X-ray diffractometer (XRD, Rigaku, Japan) with CuK radiation (λ=1.5418 Å), operational voltage of 30 kV and the current for analysis was kept at 40 mA. Fourier transform infrared (FT-IR) spectrum was recorded as KBr pellets using Varian FTS 1000 FT-IR, Mid-IR spectral range, cooled DTGS detector, Scimitar series, Varian Inc. The spectrum was analyzed by using Varian Resolution Pro version 4.0.5 from Varian. FTIR analysis of HAP powder from local Albaha cow bone residues was carried out in the range of 4,000–400 cm^{-1} to detect the presence of various functional groups. The morphology of the natural HAP nanoparticles was determined by scanning electron microscopy. In order to check the microstructure, the natural HAP powder was homogeneously scattered on carbon tape and Pt coating was made for 10 s onto the as-synthesized HAP and SEM has been taken (SEM, JEOL JSM6700, Japan) at different resolutions.

In vitro cyto-biocompatibility assay

The as-synthesized HAP nanoparticles derived from the natural bone residues have been untainted with UV radiation for 30 minutes in a biosafety chamber. Skeletal muscle precursor cells (ATCC-CRL 1772) purchased from American Type Culture Collection, were cultivated on the HAP nanoparticles. The HAP nanoparticles were placed in the bottom of a 6-well tissue culture plates after UV treatment, followed by the addition of muscle precursor cells with a concentration of 1×10^6 cells/well with the help of micropipette. The cells were propagated in Dulbecco's modified eagle medium (DMEM, pH 7.4) in which 10% fetal bovine serum and 1% penicillin–streptomycin were added. The culture plates were then held in a humidified incubator with 5% CO_2 and 95% air. The incubation temperature was set at 37 ºC. The exhausted culture medium due to cellular consumption of nutrients and dispersal of waste products was substituted with new growth medium after 3rd day and 5th day. Cell counting kit-8 (CCK-8) assay was carried out after 3 and 5 days of culture growth to evaluate *in vitro* biological property. In this study, CCK-8 assay was performed to evaluate cell viability and mitochondrial activity of skeletal muscle precursor cells seeded over the HAP. 10 µl of WST-8 (CCK-8 kit) solution was added in each well and incubated for 4 h at 37 ºC according to the given protocol of company. At the end of experiment, absorbance was calculated at 450 nm by a microplate spectrophotometer (Bio-Rad: 680, Japan). Three parallel experiments were carried out for each sample and the average reading has been produced. In order to check the morphology of cells, the cells were grown as abovementioned. The unattached cells were washed away with PBS, and attached cells were fixed in 4% formaldehyde in PBS. Finally, the cells were analyzed using a light phase contrast microscope (Olympus CK X 41 Japan) and the photos were taken by preset camera.

Results and Discussion

Preparation of HAP nanoparticles from waste bones of Albaha cows

Considering the composition of natural bone which mainly contains organic compounds (30–35%) and HAP (65–70%) on dry weight basis, we selected the local Albaha cow femur for the synthesis of natural bone precursor HAP. In the present study the preparation of natural HAP nanoparticles was done following previous techniques [3]. The main organic compound present in the natural bone was found collagen (95%). Additionally, there are other organic constituents such as hyaluronic acid, chondroitin sulfate, keratin sulfate and lipids (e.g. phospholipids, triglycerides, fatty acids, cholesterol, etc.) which are present in small quantities [2]. Femur bones taken from the Albaha cows were washed by water and acetone to remove the fats and other solid impurities. After washing, the bones have been dried at 160 °C for 2 days and processed as described in the material and methods part of this paper (Fig.1). To best of our information, no research has been done in this field in Kingdom of Saudi Arabia so far. Accordingly, our team started research in order to find out better nanotextured natural HAP material for the successful synthesis of advanced bone implants.

Characterization

The X-ray diffraction analysis of the natural extracted HAP powder was carried out which consisted of a well-crystalline phase with a hexagonal structure (JCPDS no.-86-0740). Complete crystallization of the femur bone HAP powder has been confirmed due to sharp peak intensity and well resolved peaks in XRD patterns of the samples obtained at high calcination temperature. The crystalline peaks of the as-synthesized HAP nanoparticles at $2\theta = 25.7, 31.7, 32.9, 34.1, 39.7, 46.7$ and 49.6 are evident in Fig. 2, thus confirming the presence of the HAP structure. The morphological characterizations of the natural HAP particles have been carried out by SEM taken at low and high magnifications. The micrographs of synthesized HAP particles depicted porous morphology (Fig. 3) and the natural HAP particles were found agglomerated. The size of pure HAP particles was estimated to be between 300 to 500 nm (Fig. 3a and b). EDX analysis confirmed the presence of Ca and P in the Albaha cow femur bone samples (Fig. 4), besides; oxygen has also been detected. Molecular structure of the HAP sample was characterized by FTIR (Fig. 5). The PO_4^{3-} asymmetric stretching mode of vibration has been characterized by a broad and strong band in the range of 1040 cm^{-1} and a sharp intensity peak at 960 cm^{-1} that results from symmetric stretching vibrations (Antonakos et al. 2007; Blakeslee and Condrate 1971; He et al. 2003; Lee et al. 2007; Wei et al. 2008). The sharp peaks between 450-610 cm^{-1} confirms the presence of P-O stretching vibration [1, 10, 24]. The crystalline HAP also generates characteristic OH bands at about 2930 cm^{-1}. Meanwhile, small peaks at 1650 cm^{-1} indicated the existence of a Ca–O phase. On the basis of FTIR, the formation of bone precursor HAP from the bone biowaste of Albaha was established.

Investigation of cellular biocompatibility and morphology over natural HAP nanoparticles

Cyto-biocompatibility assay of the natural HAP nanoparticles (derived from the biowaste of cow bones of Albaha city) was performed on muscle precursor cells which have been regularly maintained in cell culture division of our Laboratory. CCK-8 assay is a fine marker for evaluation of cell viability and proliferation. The test results are shown in Fig. 6a. This study (Fig. 6a) shows enhanced cellular viability on 5th day in wells having HAP nanoparticles on bottom. Figure 6(b) shows the microscopic image of control cells with media attached in one of the 6-well culture plate. Similarly Fig. 6 (c, d) illustrates the growth of cells in wells having natural HAP. Furthermore, the microscopy analysis shows uniform distribution of cells over the HAP nanoparticles at day 3 (Fig. 6c) and day 5 (Fig. 6d). The prominent growth of cells over the natural HAP nanoparticles prepared from bone biowaste validated its prospective uses in biomedical fields.

In recent times, the HAP has been extensively utilized as coatings in orthopedic and dental implants and as novel fillers for the fortification of functions of dental adhesives. HAP is a bone precursor and is commonly employed in medical fields owing to its excellent biocompatibility, osteoconductive and non-inflammatory behavior [2]. Bearing in mind the global projected demand of bone implants and in particular in Saudi Arabia, there is great need of inexpensive source of bone precursor HAP. Consequently our present research recommends making use of the novel, cost effective and environment friendly method to convert the bone bio-waste of Albaha into nano-HAP (with the aim to apply as bone implant for all age group persons in this city). Besides other significant parameters, it has also been accounted now that the nature and accessibility of biowastes are essential criteria for commercial production of natural HAP.

To sum up, in our present work, HAP powder with nano-size has been prepared from cow bones biowaste by facile extraction and heat treatment method. The obtained HAP powder was characterized by various techniques. The synthesized HAP is cyto-biocompatible. Generally sophisticated methods and expensive precursor are used for the preparation of bone implants. Herein, we report synthesis of biocompatible HAP from biowaste of bones. Therefore, the prepared HAP can be regarded as valuable resource of hydroxyapatite for bone implants. Bone weakness hampers daily activities and result in poor quality of life. The treatments are very costly. In this work we have used very simple extraction method for isolation of natural HAP from native cow bones. The success at the small scale level will finally able us to dream about the large scale production of HAP based implants for commercialization purposes under controlled conditions in Kingdom of Saudi Arabia. The whole experimental design is demonstrated in scheme 1.

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References

[1]. Antonakos A, Liarokapis E, Leventouri T. 2007. Micro-Raman and FTIR studies of synthetic and natural apatites. *Biomaterials* **28:** 3043-3054.

[2]. Barakat NA, Khalil K, Sheikh FA, Omran A, Gaihre B, Khil SM, Kim HY. 2008. Physiochemical characterizations of hydroxyapatite extracted from bovine bones by three different methods: Extraction of biologically desirable HAp. *Mater. Sci. Eng. C* **28:** 1381-1387.

[3]. Barakat NA, Khil MS, Omran A, Sheikh FA, Kim HY. 2009. Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. *J. mater. process. technol*. **209:** 3408-3415.

[4]. Bigi A, Boanini E, Rubini K. 2004. Hydroxyapatite gels and nanocrystals prepared through a sol–gel process. *J. solid state chem*. **177:** 3092-3098.

[5]. Blakeslee K, Condrate RA. 1971. Vibrational spectra of hydrothermally prepared hydroxyapatites. *J. Am. Ceram. Soc.* **54:** 559-563.

[6]. Cai Y, Tang R. 2008. Calcium phosphate nanoparticles in biomineralization and biomaterials. *J. Mater. Chem*. **18:** 3775-3787.

[7]. Cheng X, He Q, Li J, Huang Z, Chi R-a. 2009. Control of pore size of the bubble-template porous carbonated hydroxyapatite microsphere by adjustable pressure. *Cryst. Growth Des*. **9:** 2770-2775.

[8]. Cheng X, Huang Z, Li J, Liu Y, Chen C, Chi R-a, Hu Y. 2010. Self-assembled growth and pore size control of the bubble-template porous carbonated hydroxyapatite microsphere. *Cryst. Growth Des*. **10:** 1180-1188.

[9]. Du C, Wang Y-J. 2009. Progress in the biomineralization study of bone and enamel and biomimetic synthesis of calcium phosphate. *J. Inorg. Mater.* **24:** 882-888.

[10]. He G, Dahl T, Veis A, George A. 2003. Nucleation of apatite crystals in vitro by self-assembled dentin matrix protein 1. *Nature Mater.* **2:** 552-558.

[11]. Hou Z, Yang P, Lian H, Wang L, Zhang C, Li C, Chai R, Cheng Z, Lin J. 2009. Multifunctional hydroxyapatite nanofibers and microbelts as drug carriers. *Chem. Eur. J.* **15:** 6973-6982.

[12]. Hui J, Xiang G, Xu X, Zhuang J, Wang X. 2009. Monodisperse F-substituted hydroxyapatite single-crystal nanotubes with amphiphilic surface properties. *Inorg. chem*. **48:** 5614-5616.

[13]. Kithva P, Grøndahl L, Martin D, Trau M. 2010. Biomimetic synthesis and tensile properties of nanostructured high volume fraction hydroxyapatite and chitosan biocomposite films. *J. Mater. Chem*. **20:** 381-389.

[14]. Lee Y, Hahm YM, Matsuya S, Nakagawa M, Ishikawa K. 2007. Characterization of macroporous carbonate-substituted hydroxyapatite bodies prepared in different phosphate solutions. *J. Mater. Sci*. **42:** 7843-7849.

[15]. Liu J-K, Cao T-J, Lu Y, Luo C-X. 2009. Facile preparation of assembly hydroxyapatite spheres to produce nanocomposite. *Mater. Technol*. **24:** 88-91.

[16]. López-Macipe A, Gómez-Morales J, Rodríguez-Clemente R. 1998. Nanosized hydroxyapatite precipitation from homogeneous calcium/citrate/phosphate solutions using microwave and conventional heating. *Adv. Mater.* **10:** 49-53.

[17]. Ma M-Y, Zhu Y-J, Li L, Cao S-W. 2008. Nanostructured porous hollow ellipsoidal capsules of hydroxyapatite and calcium silicate: preparation and application in drug delivery. *J. Mater. Chem.* **18:** 2722-2727.

[18]. Ma MG, Zhu JF. 2009. Solvothermal synthesis and characterization of hierarchically nanostructured hydroxyapatite hollow spheres. *Eur. J. Inorg. Chem.* **2009:** 5522-5526.

[19]. Neira IS, Kolen'ko YV, Lebedev OI, Van Tendeloo G, Gupta HS, Guitián F, Yoshimura M. 2008. An effective morphology control of hydroxyapatite crystals via hydrothermal synthesis. *Cryst. Growth Des*. **9:** 466-474.

[20]. Palmer LC, Newcomb CJ, Kaltz SR, Spoerke ED, Stupp SI. 2008. Biomimetic systems for hydroxyapatite mineralization inspired by bone and enamel. *Chem. Rev.* **108:** 4754-4783.

[21]. Shum HC, Bandyopadhyay A, Bose S, Weitz

DA. 2009. Double emulsion droplets as microreactors for synthesis of mesoporous hydroxyapatite. *Chem. Mater*. **21:** 5548-5555.

[22]. Tampieri A, Sprio S, Ruffini A, Celotti G, Lesci IG, Roveri N. 2009. From wood to bone: multi-step process to convert wood hierarchical structures into biomimetic hydroxyapatite scaffolds for bone tissue engineering. *J. Mater. Chem.* **19:** 4973-4980

[23]. Tan S-H, Chen X-G, Ye Y, Sun J, Dai L-Q, Ding Q. 2010. Hydrothermal removal of Sr 2+ in aqueous solution via formation of Sr-substituted hydroxyapatite. *J. hazard. Mater.* **179:** 559-563.

[24]. Wei G, Reichert Jr, Bossert Jr, Jandt KD. 2008. Novel biopolymeric template for the nucleation and growth of hydroxyapatite crystals based on self-assembled fibrinogen fibrils. *Biomacromolecules* **9:** 3258-3267.

[25]. White AA, Best SM, Kinloch IA. 2007. Hydroxyapatite–carbon nanotube composites for biomedical applications: a review. *Int. J. Appl. Ceram. Technol*. **4:** 1-13

[26]. Wu S, Liu X, Yeung KWK, Liu C, Yang X. 2014. Biomimetic porous scaffolds for bone tissue engineering. *Mater. Sci. Eng. R:* **80:** 1-36.

[27]. Xiao J, Zhu Y, Ruan Q, Liu Y, Zeng Y, Xu F, Zhang L. 2010. Biomacromolecule and surfactant complex matrix for oriented stack of 2-dimensional carbonated hydroxyapatite nanosheets as alignment in calcified tissues. *Cryst. Growth Des*. **10:** 1492-1499.

[28]. Zhang C, Cheng Z, Yang P, Xu Z, Peng C, Li G, Lin J. 2009a. Architectures of strontium hydroxyapatite microspheres: solvothermal synthesis and luminescence properties. Langmuir **25:** 13591-13598

[29]. Zhang C, Yang J, Quan Z, Yang P, Li C, Hou Z, Lin J. 2009b. Hydroxyapatite nano-and microcrystals with multiform morphologies: controllable synthesis and luminescence properties. *Cryst. Growth Des*. **9:** 2725-2733.

Figure captions

Figure 1: Stepwise methodology adapted for the extraction of natural HAP from biowaste of cow bones of Albaha city of Saudi Arabia.

Figure 2: XRD spectrum of synthesized HAP nanoparticles

Figure 3: SEM (a, b) of synthesized HAP nanoparticles at different magnifications

Figure 4: EDX spectrum of synthesized HAP nanoparticles

Figure 5: FT-IR spectrum of synthesized HAP nanoparticles

Figure 6: CCK-8 assay (a) in which cell viability of control was 100% and comparative viability to control was expressed. Morphology of control (b), morphology of cells over HAP nanoparticles at 2 days (c), at 5 days (d) under microscope

Figure 7: Schematic illustration of plausible interaction of muscle precursor cells with natural bone precursor HAP

Figure 1

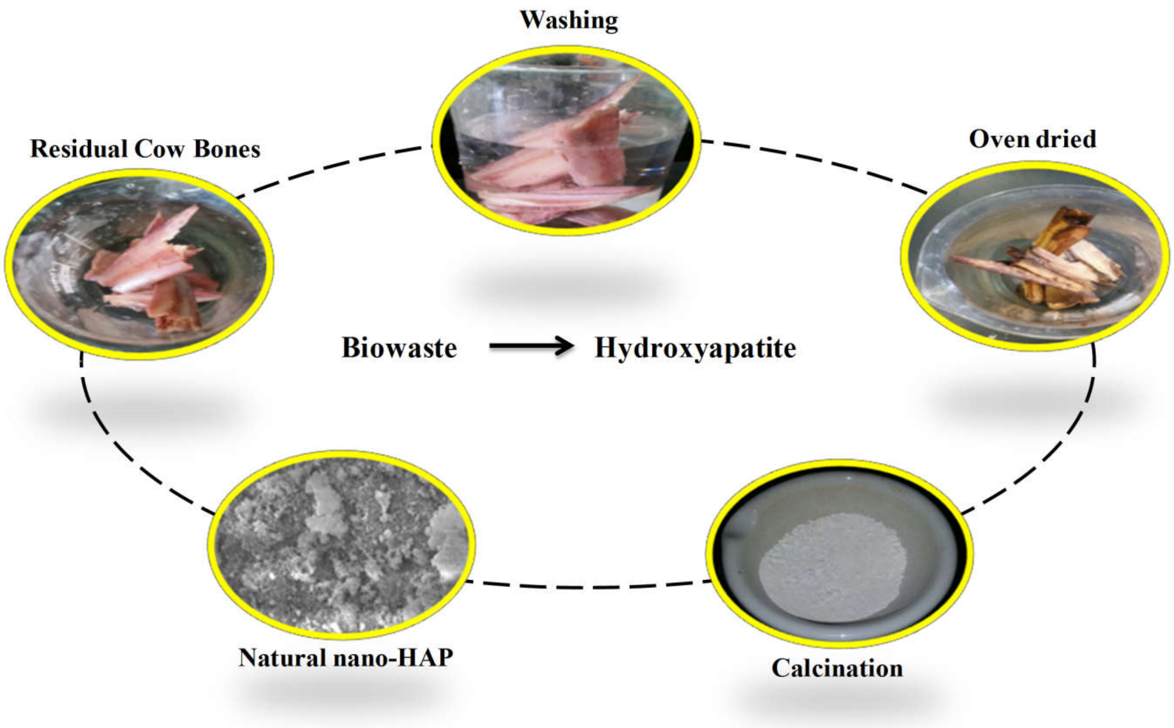


Figure 2

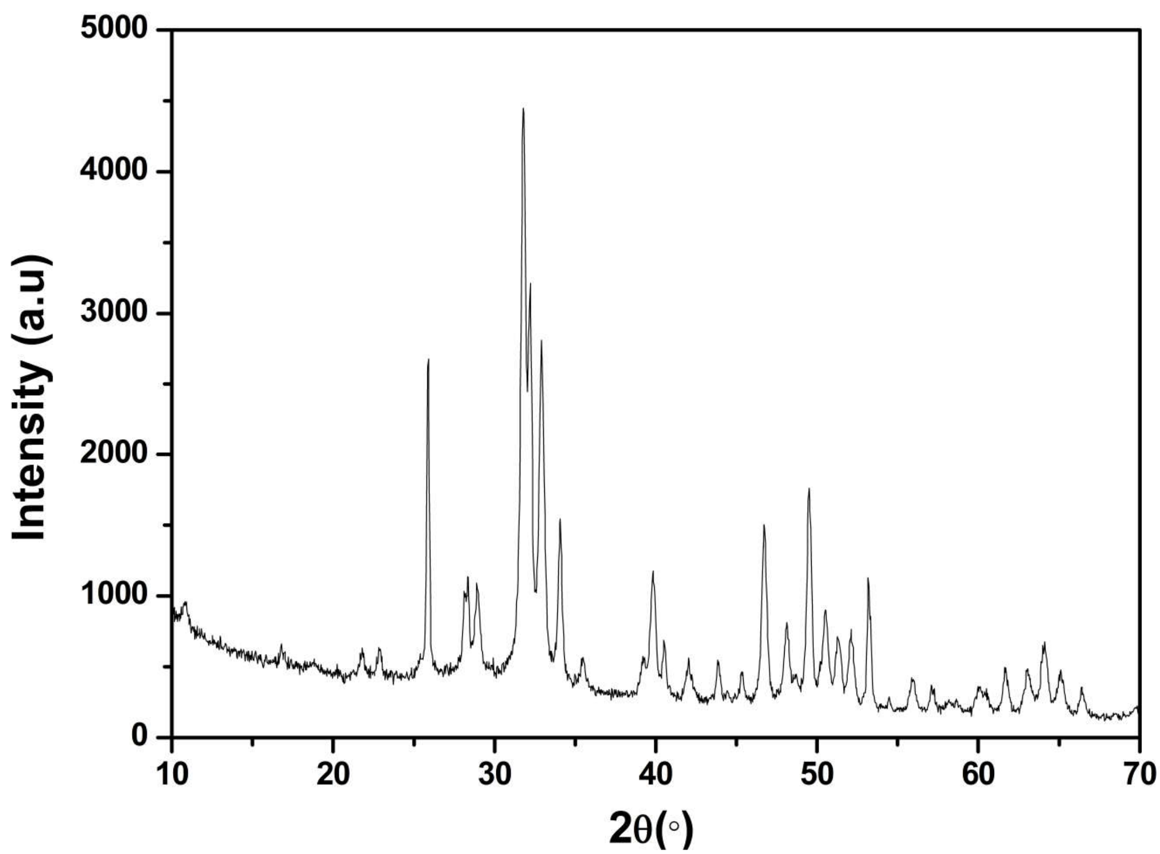


Figure 3

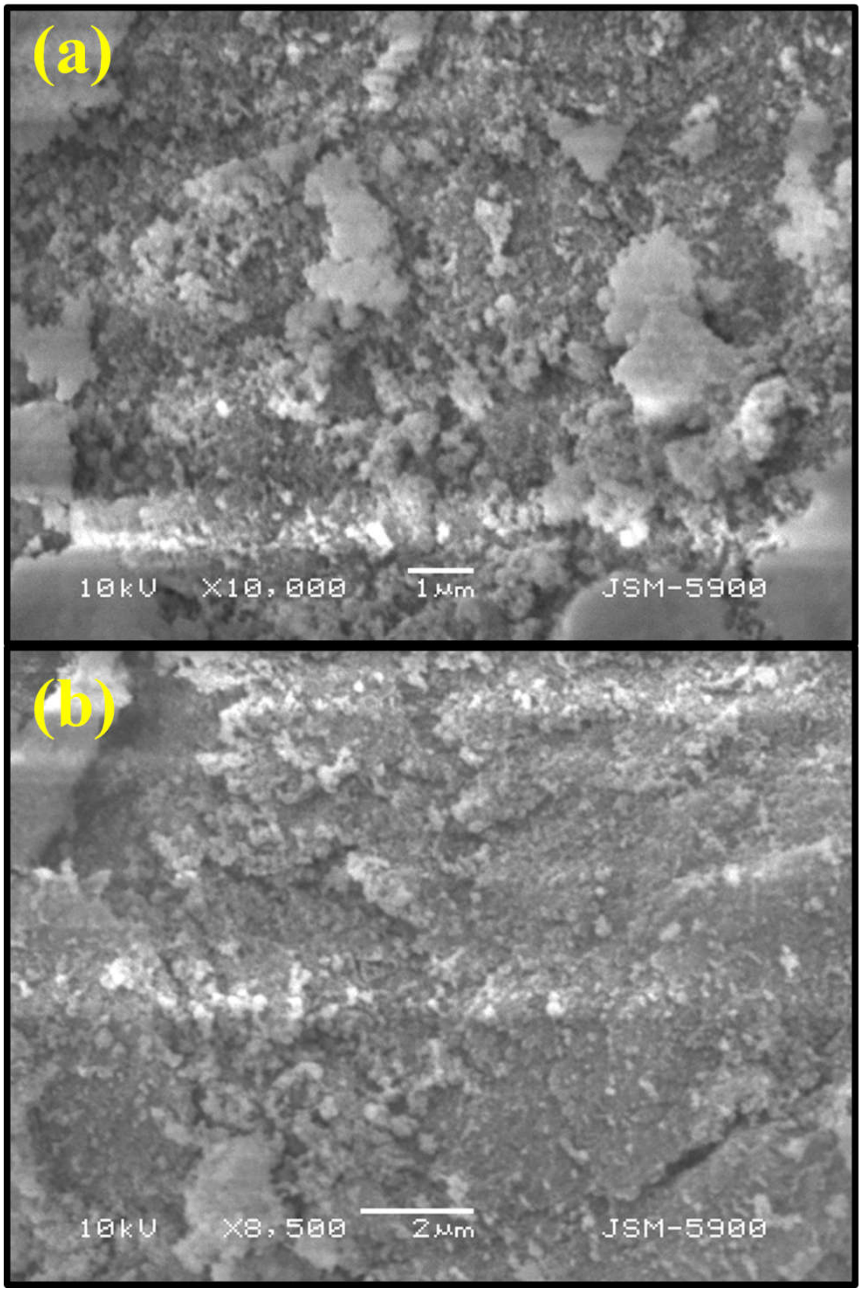
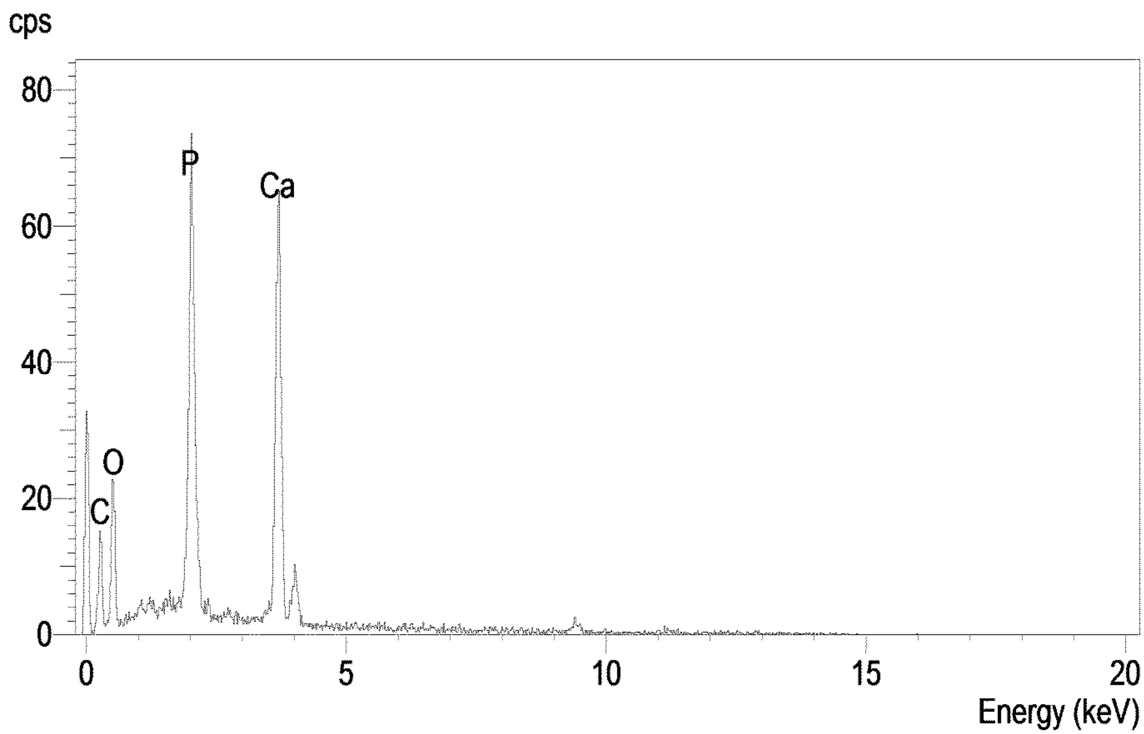


Figure 4



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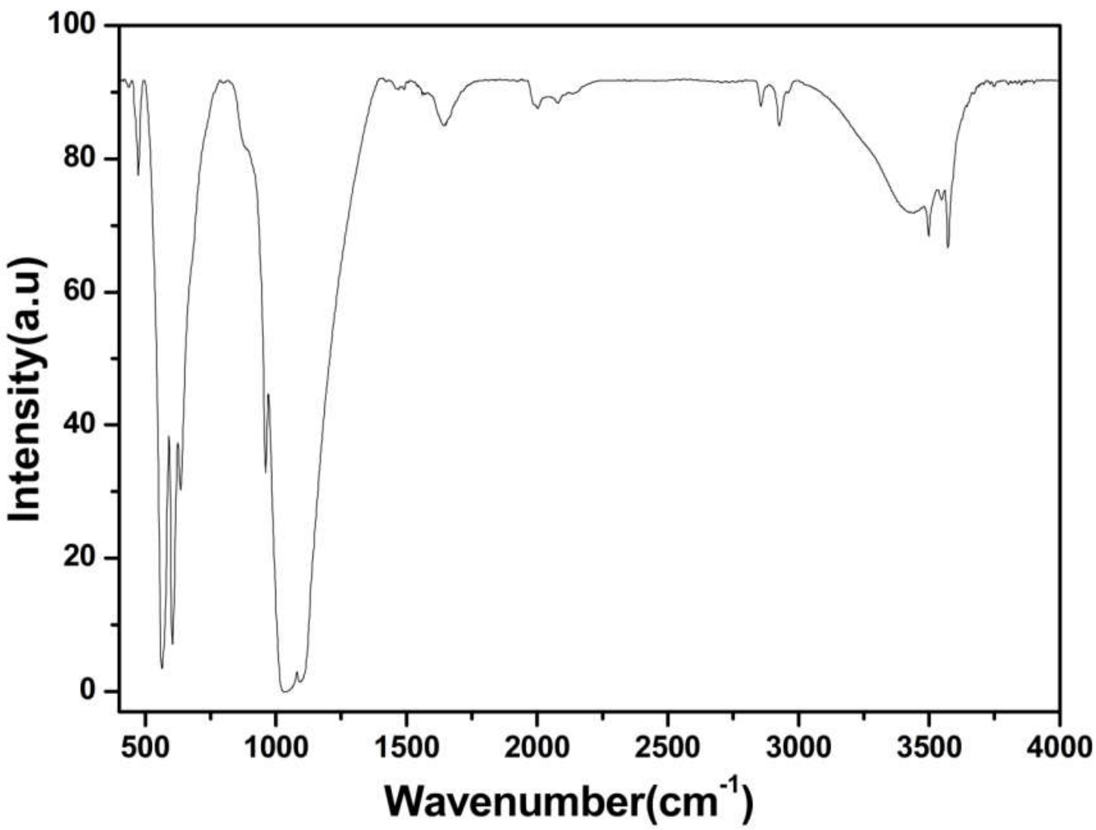


Figure 6

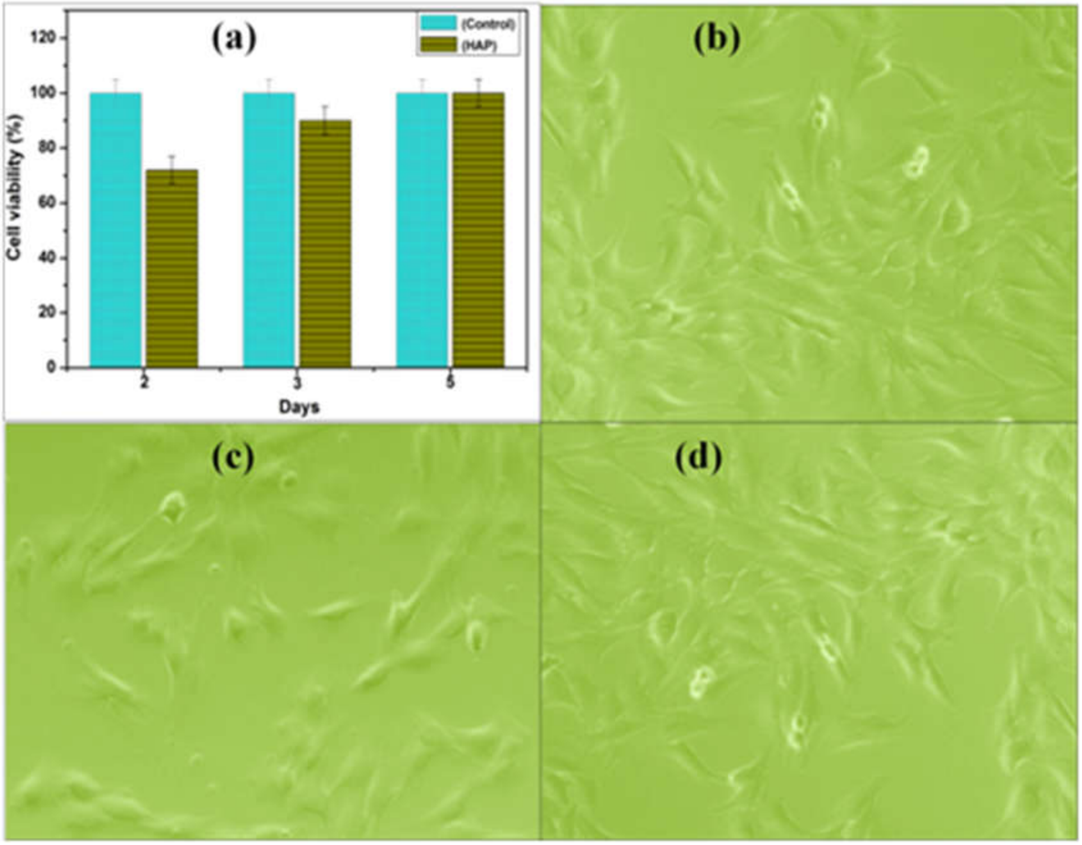
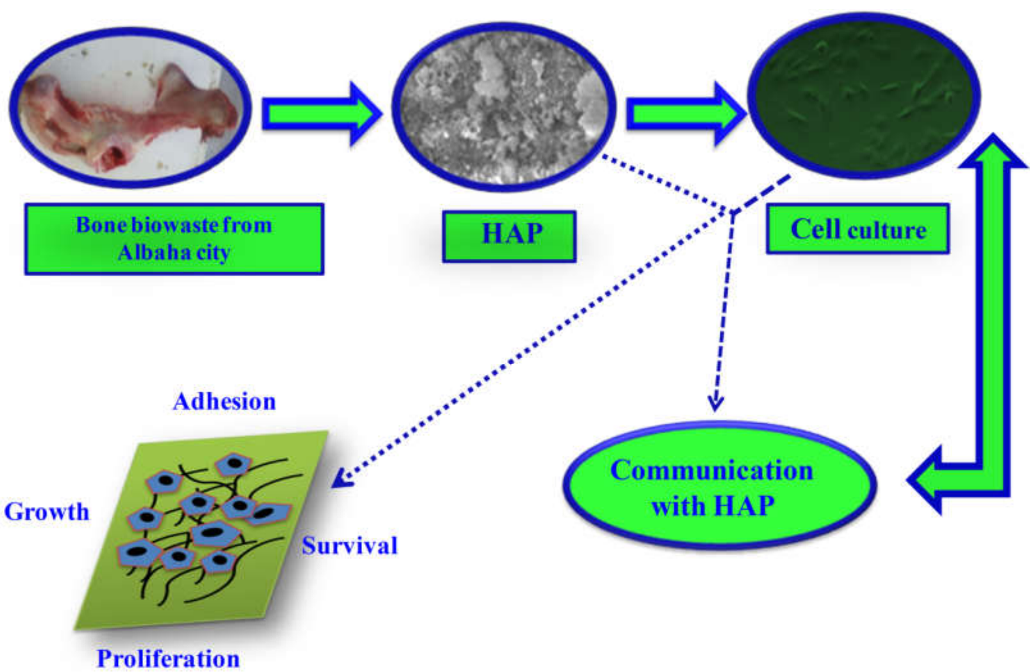


Figure 7



Short title: Bone precursor from biowaste of Saudi Arabia and *in vitro* biocompatibility

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