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Physiochemical, Morphological and Biological Characteristics of Spherical Hydroxyapatite Particles

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Abstract
Hydroxyapatite (HA) has many applications in the field of biomedical including hard tissue engineering and drug delivery. It has been used as filling material for damaged bone and augmentation. It also used to fabricatesynthetic bone graft material, scaffold and prosthesis revision surgery. In this work, HA nanoparticles were synthesized by hydrothermal method (step-1) followed by freeze drying method (step-2). The morphology of synthesized nanoparticles were analyzed by scanning electron microscope (SEM) and Transmission Electron Microscope (TEM). The spherical shape micro-nano particles of HA were produced without any surfactant using. The physiochemical properties of HA particles were analyzed by XRD and FTIR. The biological characterization includes in-vitrodegradation of HA powder that wasstudied in phosphate buffer saline (PBS) at 7.4 pHby using lysozyme.

Keywords:Hydroxyapatite, Hydrothermal, freeze drying, Lysozyme, SEM, TEM, XRD, FTIR, SBF, PBS.

Introduction
Biomaterials are the class of materials which are utilized for creating body inserts. Biomaterials produces a part or function of the human body to facilitate a secure, reliable, economic and physiologically acceptable *(Lalor et al., 1991)*. They can be metals, ceramic, polymer or glasses. Ordinarily utilized bio metals are Ti, Co or stainless steel. Due to high mechanical strength and compatibility, stainless steel is utilized in orthopedic surgery. For the repair and replacement of diseased and damaged hard tissue, bioceramics (type of biomaterials) are also used. Bioceramics can be categorized as inert (alumina, zirconia), bioactive(bioactive glass) and biocompatible (hydroxyapatite, calcium phosphate ceramics).The bioactive composites (polyethylene–hydroxyapatite), are mostly used for hard tissue engineering such as bone and teeth *(Farooq et al., 2012; Luz and Mano, 2011)*. Different methods and resources are available to produce porous hydroxyapatite with improved properties including ceramic foaming method, chemical deposition, sol-gel route, gel casting of foam, microwave processing, ceramic foaming method, polymeric sponge technique, starch consolidation and extraction from natural bones. The performance of materials used in the medical field is evaluated on their bio functionality and biocompatibility.HA coatings are utilized on these alloys to upgrade the bone holding capacity, reduce the dangerous impact of bio implant on a living creature and enhance the biocompatibility *(Kaur et al., 2015)*.HA coatings are generally applied on the metallic or alloy implants. HA is broadly utilized as the covering material since it indicates biocompatibility, bioactivity, bioresorbability and furthermore the osteoinduction (it invigorates bone development) which can fundamentally increase at nano scale *(Hench, 1993; Sadat-Shojai et al, 2013)*. HA is normally existing mineral in enamel of human teeth and the inorganic component of bone. The crystal size of HA is same to the human hard tissues in composition and morphology. The main component of HA are calcium and phosphorus, with a stoichiometric Ca/P ratio of 1.667 *(Abidi and Murtaza, 2014)*. HA can also be fabricated from normally happening waste, for example, egg shell, fish scale and animal bones. It forms a strong chemical bond with the bone tissues and thus it is perceived as a decent bone graft material *(Pandharipande and Sondawale, 2016)*. It additionally shapes nano-meter measured crystallites with various anion and cation contaminants, for instance CO_3^{2-}, F^-, Na^+ and Mg^{2+}.

Presently, HA used as a substitution for bony and periodontal imperfections, alveolar edge, middle ear inserts, tissue engineering frameworks, medicine supplier agent, dental materials and bioactive covering on metallic osseous transplant *(Sadat-Shojai et al, 2013)*. It is possible to enhance the mechanical properties of HA ceramic by controlling critical parameters of powder precursors, for example, molecule shape and size, molecule distribution and agglomeration. Nano crystalline short rod shaped HA particles <50 nm display more significant surface area *(Han, et al., 2009)*. The properties of HA can be affected by control of molecule size, chemical structure and morphology and incorporated by an assortment of techniques, for example, solid state reaction, sol-gel method and co-precipitation method *(Earl et al., 2006)*. In any case, it is hard to make the high purity HA due to calcium phosphates have many derivative and the compound of calcium phosphates strongly contingent to the response conditions. It is a significant biomaterial in the health care industry *(Chandrasekar et al., 2013)*. It is a kind of material having high adsorption limit of heavy metals, high ion exchange ability, high biological compatibility, low water solubility, high security under reducing and oxidizing conditions, accessibility and less cost. HA with various morphologies, for example, nanorods, microspheres, hexagonal crystals, and empty flowerlike structure, were produced by means of an effortless aqueous course by altering response parameters *(Sun et al., 2014)*. HA support and acceleratea number of chemical reactions that enhance the formation of bone-like apatite on the surface of implant *(K. Mori et al., 2004; Hu et al., 2004)*. HA has hexagonal structure in which calcium cation (Ca^{2+}) and phosphate anions (PO_4^{3-}) are organized around the section of monovalent hydroxide anion (OH^-). Moreover, this network of phosphate groups gives the skeletal frame work and stability *(Rajesh et al., 2012)*. The utilization of HA, in particulate structure or as nanoparticles, as a transporter for drug, protein, compound and plasmid DNA has additionally been all around archived. It used for the targeted and controlled release of drugs and other therapeutic agents are asserted to have improved bioavailability, and therapeutic response, greater efficacy and safety, controlled and delayed discharge time *(Loo et al., 2008)*.

Experimental

Materials

Analytical grade Calcium nitrate tetrahydrate, Ammonium hydroxide, di-ammonium hydrogen phosphate and procured from Sigma Aldrich Chemicals Pvt. Ltd., New Delhi.

Experimental details

Hydroxyapatite nanoparticles Synthesis

Step-1

1.00 M stock solutions of calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$, (11.807 g) and di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$, (6.603 g) were prepared using distilled water (50 mL), and subsequently diluted further to create 0.10 M solutions. By the drop-wise addition of the calcium nitrate tetra hydrate solution (50 mL) to the di-ammonium hydrogen phosphate solution, precipitate was formed with continuous stirring to get a 1.67 (Ca/P ratio) in the miscellaneous solution. The pH of the suspension resulted by the above method was ~5.1; however the pH rises to 11.0 when subjected to the drop-wise addition of ammonium hydroxide (NH_4OH) during the mixing phase. The resulting solution was vigorously stirred for further 10 minutes. Then the solution was transferred to Teflon-lined hydrothermal reactor (heated at 200°C) for 24 hours.

Step-2

The precipitate collected from hydrothermal synthesis was dispersed in petri plates and placed on -80°C in ultra-low temperature freezer (LFZ-86L) for 48 hours to solidify the sample. After that the frozen samples were located in the chamber of Lyophilizer where the ice is removed by the powerful vacuum followed by a secondary drying process which remove unfrozen water molecules and drying take place. Controlled freeze drying keeps the product temperature, low enough during the process to avoid changes in the dried product appearance and characteristics. In this phase, the temperature is raised higher than the primary drying phase,

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and can even be above 0°C, to break any physico-chemical interactions that have formed between the water molecules and the frozen material. After 48 hours the water content in the sample becomes very low (around 1-4%) and very fine powder of hydroxyapatite was collected and sealed to avoid moisture.

Characterization

X-ray diffraction

It is a powerful analytical technique used for phase identification of powder material. The crystal/particle size and unit cell dimension also observed by XRD. The interaction between sample and monochromatic ray produce diffraction when conditions satisfy Bragg's Law. At 2θ degree scanning, the possible diffraction of the lattice attained due to the random orientation of sample. The conversion of diffraction peak into d-spacing provide the phase identification in materials. The powdered hydroxyapatite sample scanned in between the range of 10 to 80 degree which provide information in the form of x-y plot (intensity vs 2θ). All the peaks and planes are indexed with *Standard Database PDF-2, Release-2013 (ICCD).*

Fourier Transmission Infra-red Spectroscopy

FTIR provide the compositional information of unknown materials (solids, liquids and gases) in the form of spectrum. The hydroxyapatite powder was measured in the frequency range of 4000cm^{-1} to 400cm^{-1} by Perkin Elmer Frontier FTIR. The resultant absorption spectrum from vibration frequencies shows the occurrence of different chemical or functional group and bonds in material. Mainly FTIR used for the identification of organic molecular compound due to the range of functional group and side chains in the infra-red range.

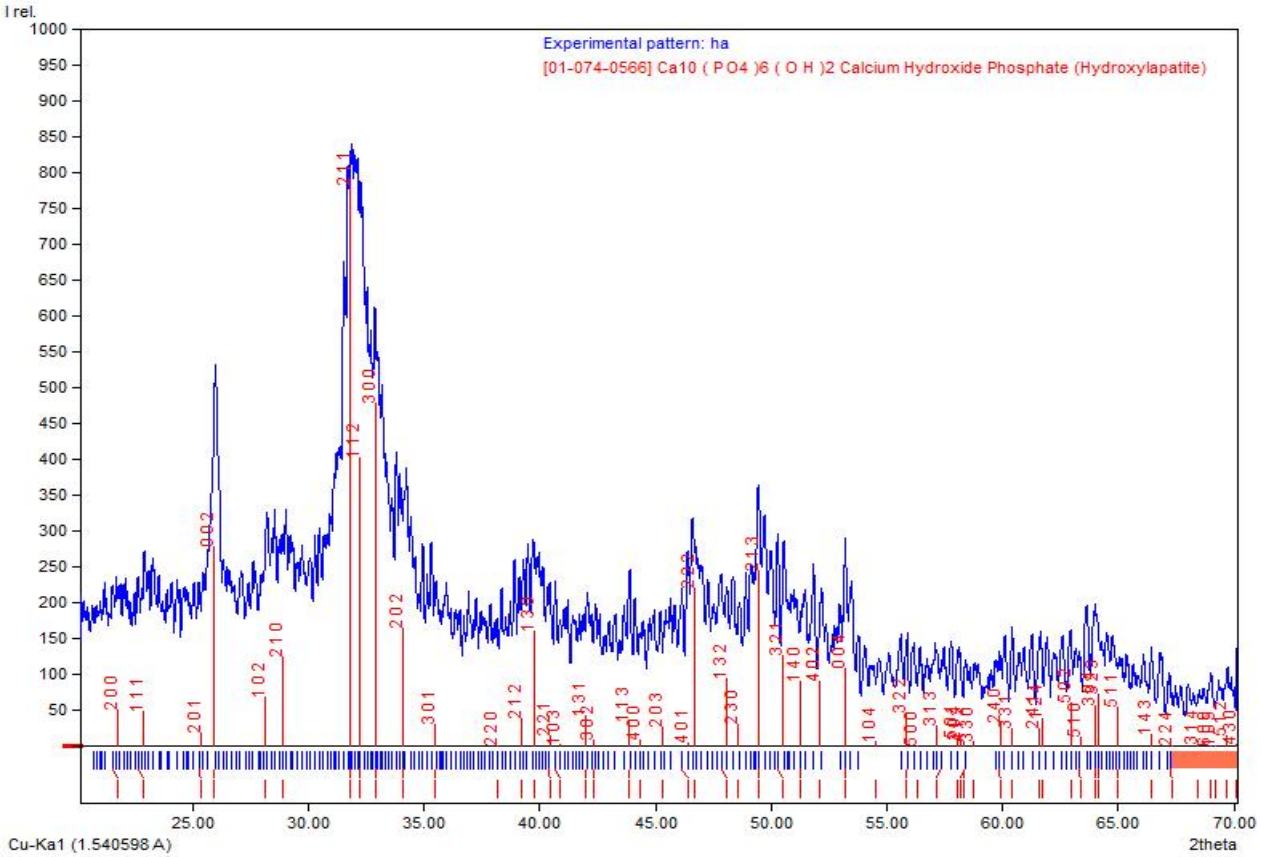

Figure 1. XRD analysis of Hydroxyapatite

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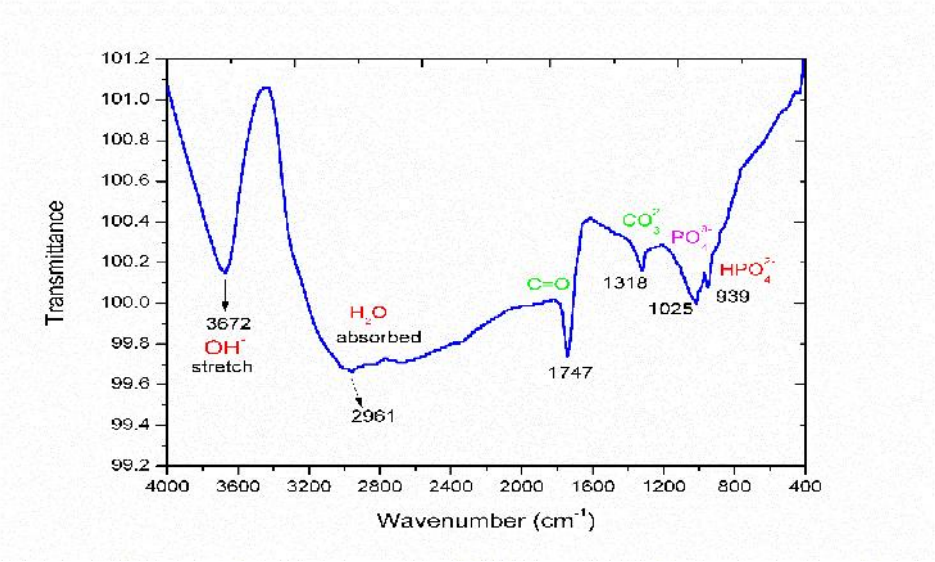

Figure 2. FTIR spectrum of Hydroxyapatite

Scanning Electron Microscopy

The lyophilized sample scaffolds were characterized for their surface and fracture sections observation. The sample scaffolds were coated with Au prior and examined by Scanning Electron Microscope (JEOL). All chitosan platforms were smooth, delicate, sponge like, adaptable and sufficiently solid to handle in wet and dry conditions without twisting. The powerful size of the pore was figured as the mean distances across of scaffold pores. No less than 12 pores were surveyed from three unique areas of a similar example. The values were expressed as the mean ± standard error.

Transmission Electron Microscopy

TEM utilize energetic charged particles to provide crystallographic, topographical, morphologic and compositional information of sample. It provide high magnification in nanometer range. It provide two dimensional black and white image of thin sliced sample. The high quality image can produced by moving electron faster, shorter wavelength. TEM offers molecular level analysis of structure and texture of sample. The good quality image provide detailed surface features, shape, size and structure of particles. The fine hydroxyapatite powder was scanned by Tecnai TEM (AIIMS, New Delhi).

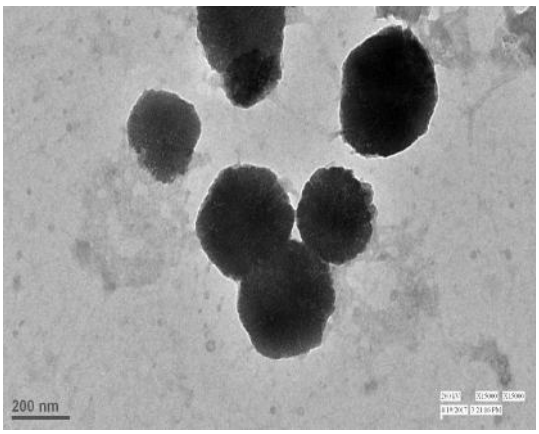

Figure 3 (a). TEM image of HA at nano scale

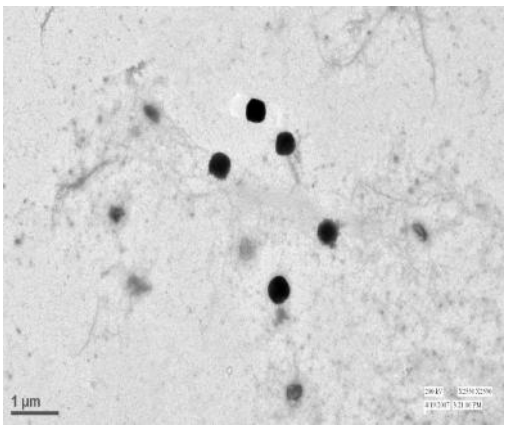

Figure 3 (b). TEM image of HA at micro scale

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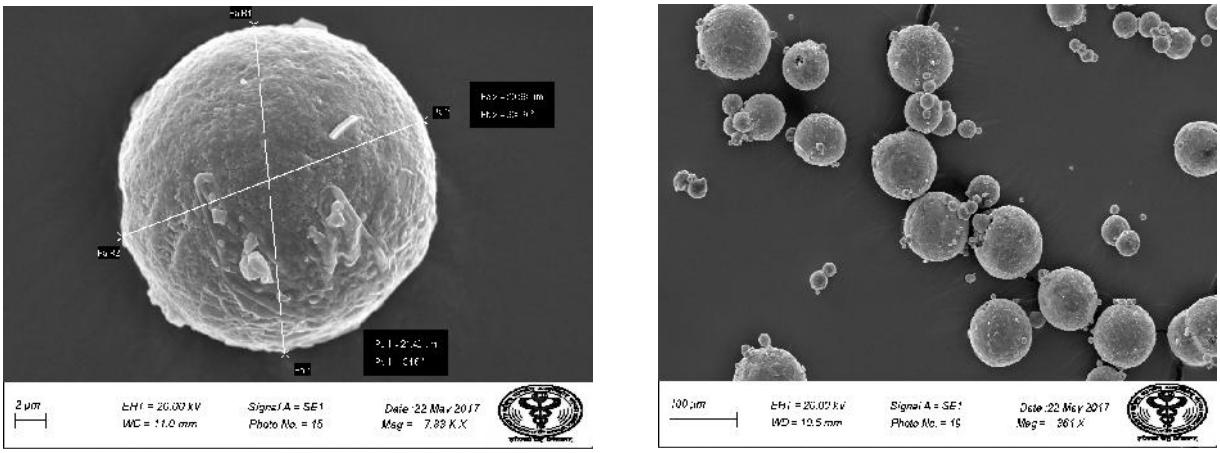

(a) **(b)**

Figure 4 (a) & (b). SEM image of HA particles at different micro scale

In-vitro degradation

The weight loss observation was used to study the degradation of powder by calculating the change in sample weight. The HA powder samples were incubated in the solution of PBS at pH 7.4 with 1×10^4 U/ml of lysozyme concentration in incubation dish and kept at 37°C for 7, 14 and 21 days. The particular concentration of lysozyme was related to the fixation in human serum. After 15 days, the samples were put out from PBS and rinsed three time with distilled water. Then the particles were dried at 50°C and weighed; the change in weight was recorded in percentage. The change in weight confirm the degradation of material that was calculated by the given formula (*Mao, 2003*):

$$W \quad htde \qquad (\%) = \frac{W_f - W_0}{W_0} \times 100$$

W_f is Final weight of sample after degradation and W_0 is Initial weight of sample.

Result and Discussion

Physiochemical Analysis

In figure 2, the major absorption peaks for PO_4^{3-} , C=O, CO_3^{2-} and OH^- groups were found in between 400-4000 cm^{-1}. The formation of HA was confirmed by various peaks present at 939 and 1025 cm^{-1} that were assigned to HPO43- and PO_4^{3-}while peak at 1318 cm^{-1} assigned to CO_3^{2-} and peaks at 2961 and 3672 cm^{-1} assigned to OH^- and peak at 1747 cm^{-1} assigned to C=O groups in HA spectrum. The band assigned from 3500-3700 cm^{-1} to hydroxyl group were showing free and stretch vibrations with strong and sharp intensity. The band from 1670-1820 cm^{-1} were assigned to C=O that was showing stretched vibration with strong intensity. The asymmetric P-O stretching was observed at 939 cm^{-1} wavelength. The band broadness indicating the presence of crystalline phase in the sample (*Tas et al., 2000*). The XRD profiles of the HA sample were good in arrangement as shown in figure 1 confirmed by Standard database PDF-2 Release-2013 (ICCD). The XRD pattern denoting strong peaks at 32° and 26° corresponding to 211 and 002 planes which confirmed the HA structure. The presence of additional peaks would change dissolution characteristics.

Morphology Analysis

The HA sample consists almost fine, homogenous and uniform particle distribution. The HA particles morphology and structure are shown in figure 3 (a & b), where the size of HA nanoparticles was found around 200 nanometers at 15000X magnification while in figure 3 (b), around 1 micrometer size particles observed at 2550X magnification. Almost spherical shaped particles observed by TEM and SEM. The SEM and TEM images shown individual separate particles at micro scale while at nanometer scale particles are slightly agglomerates. In figure 4 (b) spherical particles allowed high degree of packing. The high resolution image of SEM shown well distribution of HA micro spheres. The figure 4 (a) showing 2 micro meter diameter size of

HA particle.

Biological Analysis

The *in- vitro* degradation results reveal that the pre-freezing temperature had some effect on the deterioration of the powder by influencing the structure of the particles. The percentage degradation of HA samples were as given in table 1.

Table 1. Calculated weight loss data of HA sample

Sr. No.	Time of Incubation	% Weight loss
1.	7 days	11.6
2.	14 days	22.8
3.	21 days	29.2

Therefore, biodegradation is probably occurring along with water ion usually in volatile phases. The degradability of depends upon the degree of crystallinity, which controls the hydrolysis rate.

Conclusion

The preparation of hydroxyapatite particles by hydrothermal (Step-1) followed by freeze drying method could be the optimized process to obtaining a smaller amount of HA powders compared to using high concentration.The mono dispersed white colored hydroxyapatite particles were successfully prepared. The uniform grain size morphology of particles varies from micro to nano scale with a spherical shape. The physiochemical study demonstrate that modified shape can be prepared without using any organic surfactant and without addition of combination of temperature. The *in vitro* analysis again proved that hydroxyapatite particles are easily biodegraded by the body. The synthesized particles are inexpensive and eco-friendly that can be easily utilized in hard tissue engineering applications. The hydroxyapatite is the inorganic mineral part of hard tissue of body so HA is a promising material for various biomedical application.

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