International Journal of Scientific Research in Science and Technology
Print ISSN: 2395-6011 | Online ISSN: 2395-602X (www.ijsrst.com)
© 2021 | IJSRST | Volume 8 - Issue 1

Wet Chemical Synthesis, Characterization and Biocompatibility Study of Hydroxyapatite Used As Biomaterials

V. G. Thakare[1], V. B. Bhatkar[2], P.A.Wadegaonkar[3], S. K. Omanwar[4]

[1]Department of Physics, SPM College Nandura, Dist: Buldana (MS), India

[2]Department of Physics, Shri Shivaji College, Amravati (MS), India

[3]Department of Biotechnology, SGB Amravati University, Amravati (MS), India

[4]Department of Physics, SGB Amravati University, Amravati (MS), India

ABSTRACT

Calcium phosphate based bioceramics are promising candidates as biomaterials for tissue engineering. The wet chemical method is used for synthesis of hydroxyapatite which is calcium based bioceramic used for orthopaedic and dental applications. This paper discusses the synthesis and characterization of hydroxyapatite which has shown good *in vitro* bioactivities. Hydroxyapatite was synthesized from aqueous solutions that contain calcium nitrate and di-ammonium hydrogen orthophosphate. X-ray diffraction identified HAp as crystalline phase with hexagonal structure. X-Ray diffraction of HAp is in good agreement with the standard of lattice constant a = b = 9.418 Å, c = 6.884 Å with space group P63/m. The Fourier transform infrared spectra of the sintered HAp shows the absorption bands characteristic to hydroxyapatite. Scanning electron microscopy revealed that the surface morphology was spherical with particle sizes in range of micrometer. The hydroxyapatite was evaluated for the biocompatibility characteristics such as antimicrobial activity, cytotoxicity and biodegradation.

Keywords: Hydroxyapatite, Bioceramic, Wet Chemical, Antimicrobial Activity, Cytotoxicity, Orthopaedic.

I. INTRODUCTION

Tissue engineering offers a new approach to regenerate diseased or damage tissues such as bone (Rezaei et al. 2014). The use of bone-substituted materials in the science of biomaterials is an important objective due to their bioactive and biocompatible properties. Calcium phosphates, major components of natural bone, have bioactive and biocompatible properties. Therefore calcium phosphates have been used in an interdisciplinary field of science involving chemistry, biology, medicine, dentistry and geology for over 20 years. Among calcium phosphate ceramics tri-calcium phosphate ($Ca_3(PO_4)_2$,TCP), tetracalcium phosphate ($Ca_4P_2O_9$, TetCP), and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) have been studied for application in medical fields. The only TCP, a resorbable material and HAp, a bioactive ceramic, that induces bone formation on its surface, are mainly used as bone-substituted materials (Cengiz et al. 2008; Dorozhkin et al. 2007; Zhou et al. 2013).

Hydroxyapatite (HAp) ceramics have been recognized as substitute materials for bone and teeth in orthopedic and dentistry field due to their chemical and biological similarity to human hard tissue (Yarosh et al. 2001). More over HAp has been recognized as a

bioactive material having the direct bonding capability to the surrounding tissues therefore it has an excellent biocompatibility with human teeth and bone, making it very attractive for biomedical applications (Quan et al. 2008; Bouyer et al. 2000; Jillavenkatesa et al. 1998 Silva et al. 1997). The greatest potential for bone substitution is shown by materials based on hydroxyapatite (HAp), which can develop tight bonding with bone tissue, exhibits osteoconductive behavior, is stable toward bioresorption, and has no adverse effects on the human organism (Orlovskii et al. 2002).

In literature, so many methods have been reported for synthesizing HAp, including Sol-gel (Dou et al. 2012), co-precipitation reaction (Shen et al. 2010), mechanochemical synthesis (Mostafa et al. 2005), The Chemical precipitation process is the most reported method for preparing HAp particles. This process is simple, low cost and suitable for industrial production. In this research, we have used a simple aqueous precipitation process that could produce HAp crystalline powder at a relatively low temperature, with a fairly short synthesis time. We have also assessed cytotoxicity, antimicrobial and biodegradation behavior of this ceramic. This paper presents the synthesis and characterization of pure crystalline HAp ceramic and their biocompatibility in detail.

II. METHODS AND MATERIAL

2.1. Materials and methods

The hydroxyapatite powders were prepared by a simple aqueous wet chemical method. Calcium nitrate $Ca(NO_3)_2{\cdot}4H_2O$ and di-ammonium phosphate $(NH_4)_2HPO_4$ were dissolved in deionized water at concentrations. Then pH value of the solution was adjusted to 10–11 with an ammonia (NH_4) solution. The $(NH_4)_2HPO_4$ solution was mixed with the $Ca(NO_3)_2$ solution drop by drop under continuous stirring heating at 40^0c. HAp precipitation by the following reaction:

$$10Ca(NO_3)_2{\cdot}4H_2O + 6(NH_4)_2HPO_4 + 8NH_4OH = Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 20H_2O$$

After stirring for 4 h, the precipitates were centrifuged, rinsed with deionized water 3 times, dried at 80^0C for 24 hrs, After drying a dried sample was obtained this was crushed to obtained powders. The powders were then compacted into pellets with the help of die and punch in a hydraulic press at a load of 4 Ton for 90 seconds with a diameter of 10 mm and thickness of 2 mm. After the pellets were sintered at 900^0C for 2hrs to improve their resistance, they were degreased, ultrasonically cleaned, and sterilized in a steam autoclave at 120^0C for 30 min.

2.2 Biocompatibility screening

Cell culture studies

The cell culture study was done in direct contact method (ISO 10993–5, 1999) using normal human fibroblast cells 3T3. The test samples were prepared by setting the powder in the form of discs of 10 mm diameter and 2 mm thickness by using KBr pellet machine. They were cleaned ultrasonically and sterilized by autoclaving.

The culture medium consisted of Eagle's MEM (containing glutamine) and fetal bovine serum (10%) along with antibiotics. First the pellet seeded into 24 well dishes then Human fibroblast cells (3T3, supplied by NCCS, Pune) were sub-cultured and seeded on pellet into the 24 multiwell dishes to form a monolayer and incubated at 37°C under 5% CO_2 for 24 hrs. The morphology of the cells of the material was examined under a phase contrast microscope. Test was done in duplicate, along with control samples.

Antimicrobial Activity

Antibacterial property of HAp sample was investigated using the quantitative viable count method. The stock solution was prepared by mixing 1mL *E. coli* with 9 Ml of LB (Luria- Bertani) broth and incubated at 37^0 C for 24 h with shaking at 250 rpm. The broth was then serially diluted upto 10^{-6}. The

dilutions of 10^5 and 10^6 were used as working concentrations. 0.01g HAp powder was autoclaved and mixed with the stock solution of dilution 10^5 and 10^6. 0.1mL of the prepared mixture was then inoculated on LB agar plates followed by incubation at 37^0 C for 24 h. Finally, the number of colony-forming units was counted for each dilution.

Test of Biodegradation

Biodegradation test of HAp pellet was done by taking Tris-HCl buffer solution. 0.05M Tris- HCl Solution was prepared using distilled water. The pH of solution was maintained 7.4 at 37^0c by adding 1M HCl. HAP pellet was soaked in Tris-HCl buffer solution, the samples was dried at 120^oc after every one week and final weight of sample was taken. This process was repeated for five weeks.

$$\%\text{Weight loss} = \frac{W_0 - W_t}{W_0} * 100$$

Where, W_0 = initial weight of sample

W_t = final weight of sample after soaking in Tris-HCl solution

2.3 Powder Characterization Techniques

X-ray powder diffraction (XRD) technique was used to study the effect of calcination temperature on the phase evolution and phase identification. The dried powder obtained after heat treatment at 85 °C and the calcined powder at 900 °C were ground into fine powder using a mortar and pestle to breakdown the powder agglomerates before analyzing in an X-ray diffractometer. Powder samples were placed in the specimen holder of a Rigaku diffractometer (XRD, miniflex Rigaku), and then analyzed, using Ni-filtered CuKα radiation (λ = 0.1542 nm) in the step scanning mode, with tube voltage of 40 kV and tube current of 40 mA. The XRD patterns were recorded in the 2θ range of 20 to 70°, with a step size of 0.02° and step duration of 1 s.

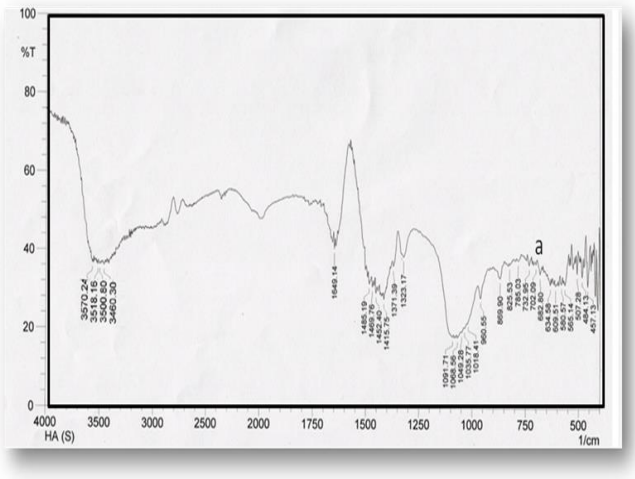
The FTIR spectra of the samples were recorded in a wave number range of 450 –4000cm^{-1} using a Fourier Transform Infrared spectrophotometer (SHIMDZU IR Affinity 1 FTIR Spectrometer).

Scanning electron microscopy (SEM) technique was also used to observe the particle-size and agglomeration of the as-synthesized HAp powder calcined at 900 °C. For this, a very small amount of powder was placed on an adhesive carbon tape, coated with gold/palladium and then observed in a FE-SEM (HITACHI S-4800).

III. RESULTS AND DISCUSSION

FTIR analysis

The presence of OH and PO_4 functional groups was confirmed by the FTIR spectrum. FTIR spectra of the prepared and calcinated samples are given in Fig. 1a and b. Peak at 1629 cm–1 belongs to the bending mode of OH group while the sharp peak at 3570 cm–1 denotes the OH stretching vibrations. The broad peak around 3432 cm^{-1} is due to water molecules. All the phosphate modes (962 cm^{-1} -υ_1, 495 cm^{-1}- υ_2, 1082 cm^{-1} and 1091 cm^{-1} - υ_3, 561 cm^{-1} and 603 cm^{-1} -υ_4) are present. The peaks at 1456 cm^{-1} and 1415 cm^{-1} are due to the carbonate, which might be incorporated from the atmosphere to these samples during the preparation (Jaworski et al. 2009).



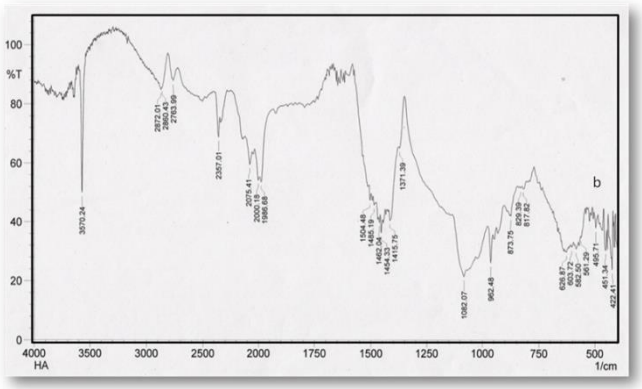


Fig 1: FTIR spectra of HAp (a) as prepared (b) calcinated.

XRD analysis

The structural analysis of sample was done by the powder X-ray diffraction. The XRD patterns of the synthesized and calcinated samples are shown in Fig. 2a and b. The XRD pattern of calcinated sample shows sharper peaks which indicate better crystallinity. The peak positions are in good agreement with the ICDD file number (01-072-1243) having lattice parameters a≈b≈0.9418 nm, c≈0.6884 nm and space group P63/m (176). Thus, standard HAp with hexagonal structure is formed during synthesis, which remains stable after the calcinations. The investigated powders show no secondary phases such as tricalcium phosphate and calcium oxide.

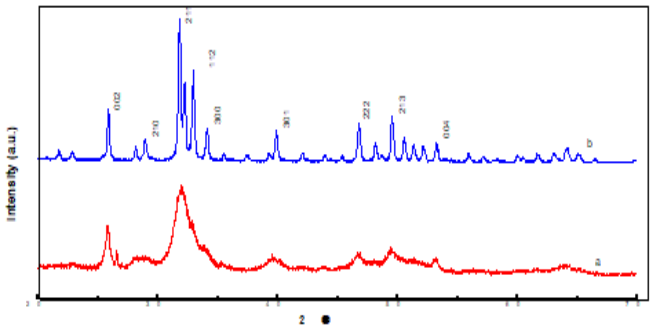


Fig. 2 XRD patterns of HAp (a) as prepared (b) calcinated

Morphology

The surface morphologies of as prepared and calcinated sample were investigated by FESEM, as given in Fig. 3. Fig. 3a shows FESEM image of as prepared powder of HAp consists of agglomerated which are composed of fine crystallites and fig. 4b and 4c shows FESEM images of calcinated powder of HAp shows that spherical-like shape with mean particle sizes 41.20 nm. This shows the agglomeration it is due to heating.

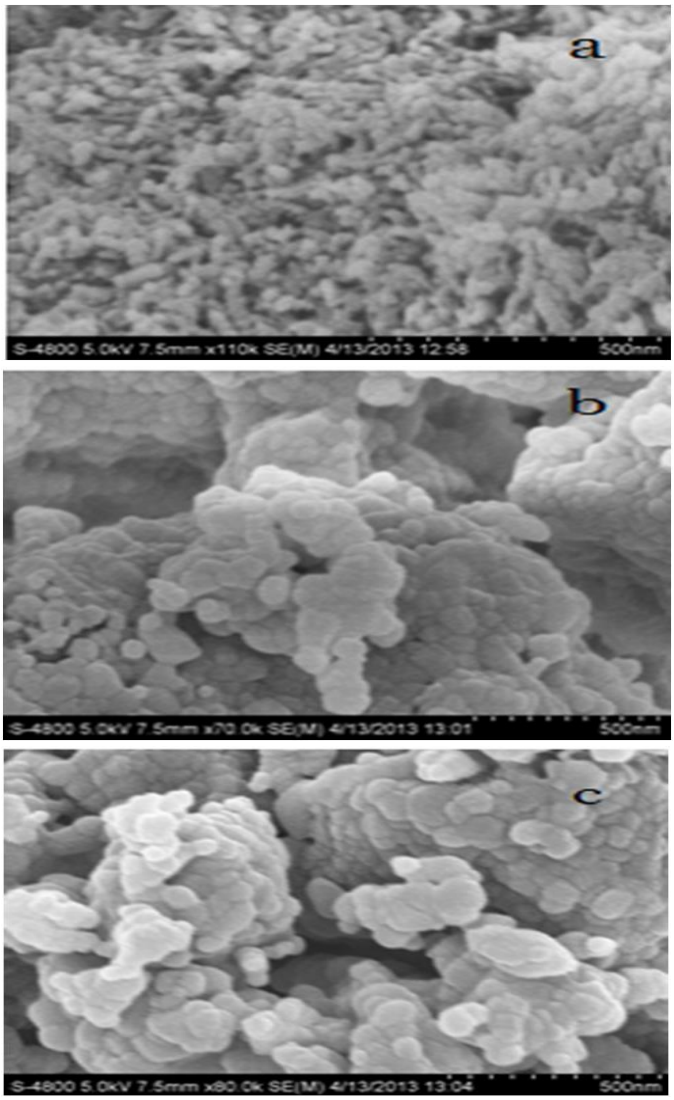


Fig.3 FESEM image of HAp a) as prepared b) calcinated

Cytotoxicity

The cytotoxicity of the HAp pellets was evaluated with mouse fibroblast 3T3 cell line. The cells were grown in presence of HAp pellets for 24, 48 and 72 hrs in 24 well plates. cell proliferation was observed under phase contrast microscope as shown in fig.4 The phase contrast microscope images in Fig.4 (a,b) shows the live cells near HAp pellets after 24, 48 hrs of growth. The cells in both the images show a typical

elongated shape characteristic of live fibroblast, suggesting the presence of normal cell growth behavior and absence of cytotoxicity. After 72 hrs of culture cells became more confluent as shown in Fig.4.d. After 74 hrs of growth, the cell population increased significantly near HAp pellets surface and (M) area of HAp pellet as shown in fig4.d. Increasing numbers of fibroblast cells with time suggests an increase in cell proliferation and/or survival near HAp pellets surface. Thus, the presence of HAp pellets does not negatively affect the fibroblast cells.

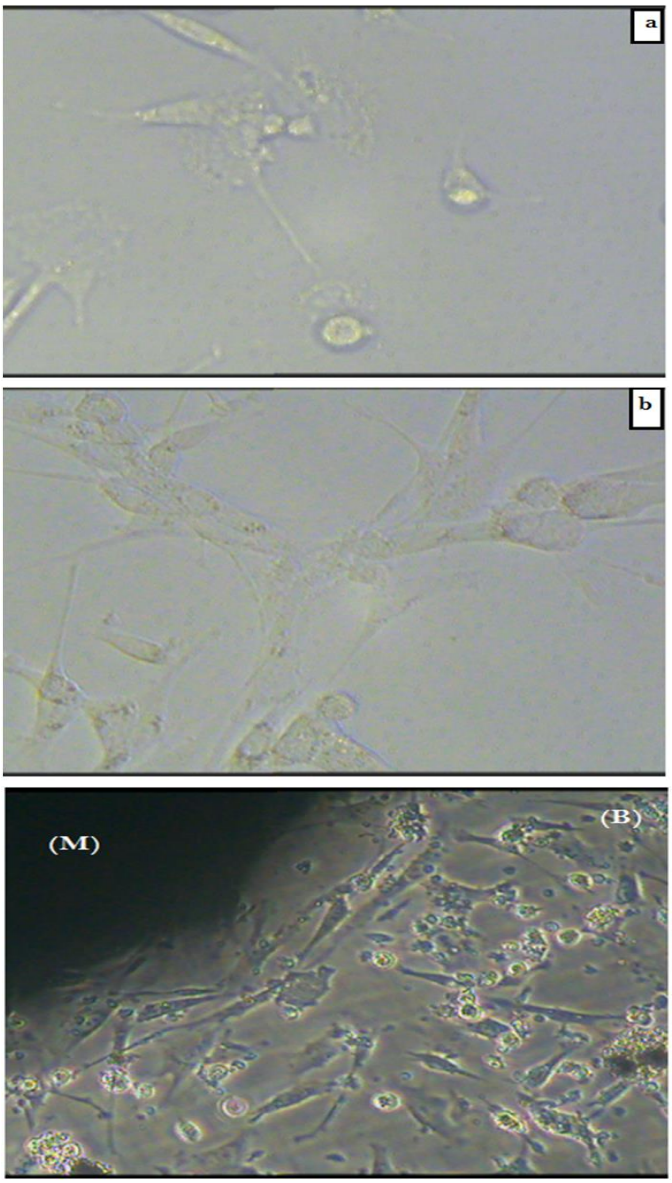


Fig. 4. 3T3 mouse fibroblasts (a, b, B) were cultured in DMEM for 24, 48, 72 hrs on pellets of HAp observed by microscope. (M) Area of pellets of HAp

Antimicrobial Activity

Antibacterial property of HAp samples calcinated at 900^{0}c was investigated using quantitative viable count method. 0.01gm powder of HAp was autoclaved at 120^{0}c for 30 min. Then this powder was incubated with *E. Coil* suspension for 24 hrs as shown in fig. 5. In this study agar plates a and b were control plates (in the absence HAp powder) with $10^5$and 10^6 times dilution of *E. Coil* culture and plates c and d shows the decrease in the number of colonies of *E. Coil* in the presence of 0.01gm powder of HAp. HAp powder shows significant antibacterial effect.

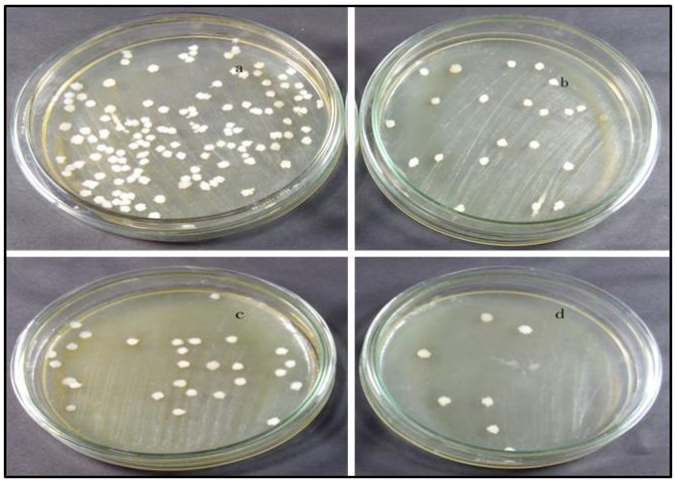


Fig. 5 a and b control 10^5 1nd 10^6 times dilution of *E. coli* culture

Fig. 5 c and d 0.01g HAp powder with 10^5 1nd 10^6 times dilution of *E. coli* culture

In-Vitro degradation:

Degradation of HAp pellet sample was carried out in Tris-HCL solution. HAp samples were soaked in Tris buffer solution at pH 7.4 and temperature 37°C for four week. When the HAp pellet was soaked in Tris buffer solution, the pH of buffer increases from 7.4 to 7.8 which confirms the biodegradation of HAp. The weight loss of the HAp pellet was approximate 1.06% in five week as shown in fig 6.

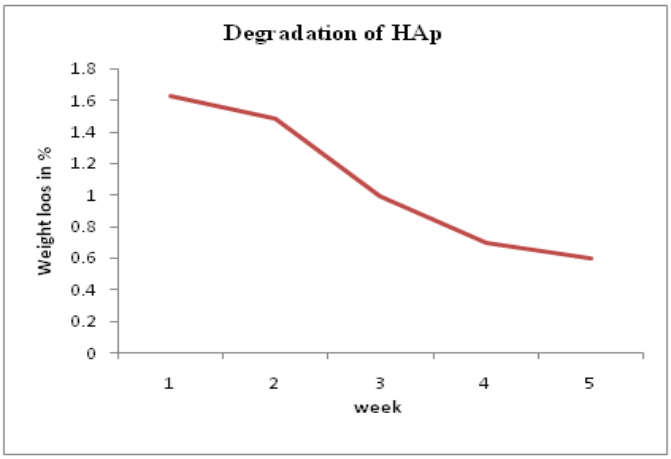


Fig: 6 Degradation of HAp

IV. CONCLUSION

The present study reveals that HAp powder can be synthesized by aqueous wet chemical method by using calcium nitrate and di-ammonium hydrogen orthophosphate. Crystalline HAp has been achieved by simple calcination at 900°C (for 2 hrs). FTIR, X- ray diffraction indicated the phase purity and crystallinity of the HAp powder. FTIR, XRD studies confirm the formation hexagonal structure of HAp. Moreover, the HAp particles showed antibacterial activity against *E. coli.* The weight loss of the HAp pellet was approximate 1.06%.

Cell culture, cytotoxiciy assays and staining show that HAp has no cytotoxic effect on cells and

Possess good biocompatibility. Cell viability and cell attachment studies proved the non-toxic nature of these scaffolds with enhanced cell attachment. All these results essentially suggest that hydroxyaptite can be superior candidates for bone tissue engineering.

V. REFERENCES

[1]. Rezaei A, and Mohammadi MR, (2012) Development of Hydroxyapatite Nanorods–Polycaprolactone Composites and Scaffolds Derived from a Novel In-Situ Sol-Gel Process. Tissue Engineering and Regenerative Medicine 9:295-303.

[2]. Cengiz B, Gokce Y, Yildiz N, Aktas Z, (2008) Synthesis and characterization of Hydroxyaptite nanoparticles. A Colloids and Surfaces A: Physicochem. Eng. Aspects 322:29–33

[3]. Dorozhkin SV (2007) calcium orthophosphates. Journal Mater Science 42:1061–1095.

[4]. Zhou S, Ma J, Shen Y, Haapasalo M, Ruse N, Yang Q, Troczynski T, In vitro studies of calcium phosphate silicate bone cements. (2013) Journal Mater Science: Mater Med 24:355–364.

[5]. Yarosh EB. Dmitrevskii BA, Naryzhnyi VP, Tsvetkov SK (2001) Some Characteristics of Synthetic Hydroxyapatite. Russian Journal of Applied Chemistry 74:1058-1060.

[6]. Quan R. Yang D. Wu X. Wang H. Miao X. Li W, (2008) In vitro and in vivo biocompatibility of graded hydroxyapatite–zirconia composite bioceramic. Journal Mater Science: Mater Med 19:183–187.

[7]. Bouyer E, Gitzhofer F, Boulos MI, (2000) Morphological study of hydroxyapatite nanocrystal suspension. Journal of materials science: materials in medicine 11: 523-531.

[8]. Jillavenkatesa AR, (1998) sol-gel processing of hydroxyapatite. Journal of materials science 33: 4111 – 4119.

[9]. Silva VV, Domingues RZ, (1997) Hydroxyapatite–zirconia composites prepared by precipitation method. Journal of materials science: materials in medicine 8:907- 910.

[10]. Orlovskii VP, Komlev VS, Barinov SM, (2002) Hydroxyapatite and Hydroxyapatite-Based. Ceramics Inorganic Materials 38:973–984.

[11]. Dou Y, Cai S, Xu G, Hu H, Ye X, (2012) Preparation of mesoporous hydroxyapatite films used as biomaterials via sol–gel technology. Journal Sol-Gel Science Technology 61:126–132.

[12]. Shen SC, Chia L, Ng WK, Dong YC, Tan BH, (2010) Solid-phase steam-assisted synthesis of hydroxyapatite nanorods and nanoparticles. Journal Mater Science 45:6059–6067.

[13]. Mostafa NY, (2005) Characterization, thermal stability and sintering of hydroxyapatite powders prepared by different routes Materials. Chemistry and Physics 94 333–341.

[14]. Andiappan M, Sundaramoorthy S, Panda N, Meiyazhaban G, Winfred SB, Venkataraman G, Krishna P, (2013) Electrospun eri silk fibroin scaffold coated with hydroxyapatite for bone tissue engineering applications. Progress in Biomaterials 2(6):1-11.

[15]. Jaworski R, Pierlot C, Pawlowski L, Bigan M, Martel M, (2009) Design of the synthesis of fine HA powder for suspention plasma sparaying. Surface & Coatings Technology 203:2092–2097.