2.1 INTRODUCTION

Hydroxyapatite [HAP, Ca_{10} (PO_{4})_6 (OH)_2] has assumed substantial interest and importance because of its chemical and crystallographic similarity to natural calcium phosphate mineral present in the natural bone. Considering the numerous applications of calcium phosphate compounds in biomedical fields, several HAP synthesis techniques have been developed. The most commonly used technique for the formation of HAP is the precipitation technique, involving wet chemical reactions between the calcium and phosphate precursors under controlled temperature and pH conditions (Jarcho et al; 1976, Slosarczyk et al; 1996 and Kweh et al; 1999). However, this method has some inherent disadvantages, primarily the difficulty in controlling of the pH value above 9 to avoid the formation of a Ca-deficient HAP which on sintering undergoes easy decomposition, forming tricalcium phosphates.

Nanosized HAP exhibits much higher bioactivity than coarse crystal and the resultant nanophase ceramics represent a promising class of orthopedic and dental implant applications with improved osseointegrative properties (Bezzi G et al; 2003 and Lui J et al; 2003). On the other hand, nanosized HAP can provide large interfaces, resulting in high catalytic activity and great adsorption capability in catalysis and separation fields. These applications stimulate researchers to develop new synthesis methods of HAP nano materials.

This chapter elaborates the various methods/techniques adapted to synthesis nanostructured hydroxyapatite. The methods are as follows:

1) Sol-gel technique
2) Co-precipitation method
3) Co-precipitation method assisted with ultrasonic irradiation technique
The *in-vitro* anti-proliferative activity of fluorapatite is discussed in this chapter.

2.2 SOL-GEL TECHNIQUE

2.2.1 INTRODUCTION

Hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, HAP) is the bioceramic material consists of calcium and phosphate minerals (Murugan et al; 2004). However, densified HAP has low mechanical strength and fracture toughness compared to natural human bone and it has restricted their practical applications (Shareef et al; 1993). HAP promotes faster bone regeneration, and direct bonding to regenerated bones without intermediate connective tissues (Mobasherpour et al; 2007). The sol-gel technique is an elective method for the synthesis of highly pure HAP powder due to the possibility of systematic control of the process parameters. This method offers a molecular mixing of calcium and phosphate which is capable of improving the chemical homogeneity and comparatively low synthesis temperature. This present work discuss about the synthesis of nanostructured hydroxyapatite by water based sol-gel method.

2.2.2 MATERIALS AND METHODS

Calcium nitrate tetra hydrate (Ca(NO$_3$)$_2$.4H$_2$O), di-ammonium hydrogen phosphate ((NH$_4$)$_2$HPO$_4$) and ammonia solution were used to synthesis HAP nano particle, these analytical grade chemicals are purchased from Merck used without any further purification. 1M of calcium nitrate and 0.6M of di-ammonium hydrogen phosphate dissolved in double distilled water in room temperature. Phosphate solution was added with drop by drop in calcium nitrate solution at temperature 75°C and pH was maintained at 11 for throughout the experiment using ammonia solution. The resultant sol-gel was continuously stirred at a temperature 75°C for 12 h at a constant pH of 11 and this product was allowed to cool for 24 hours and then aged gels washed with double distilled water and ethanol at least three times. Finally, sol-gel was kept in hot air oven at 85°C for overnight, this experimental method as shown in Fig. 2.2.1.
Synthesized HAP powder characterized by XRD, FTIR and SEM to determine the fraction of crystallinity, crystallite size of the HAP sample using powder X-ray diffraction instrument D8 advanced BRUKER, Spectrometer using CuKα radiation source and its wave length (λ=0.15405 nm), data collected from the 2θ range from 20° to 60° in steps of 0.019° and count time 0.2S. The crystallite size of the sample was calculated from the Scherrer’s equation (Pang et al; 2003)

\[ X_s = \frac{0.9\lambda}{\beta\cos\theta} \]  

(2.1)

Where \( \beta \) is the full width half maximum under considerations of selected diffraction maximum intensity peaks in radian, \( \lambda \) is the wave length CuKα radiation source (\( \lambda=0.154 \) nm) and \( \theta \) is the angle of diffraction (°). The fraction of crystallinity \( (X_c) \) of the HAP nanoparticle calculated from the equation (Landi et al; 2000)

\[ X_c = (0.23/\beta)^3 \]  

(2.2)

Fourier transform infrared spectroscopy (FTIR) spectrum was recorded in the range of 4000-400 cm\(^{-1}\) by using an instrument Perkin Elmer RXI FT-IR spectrometer by KBr pellet technique. The morphology of synthesized HAP sample was viewed by the Quanta 200 FEG scanning electron microscope (SEM) magnification range from 12x to greater than 1,00,000x

2.2.3 RESULTS AND DISCUSSION

This process can be correctly named as sol-gel combustion method. The XRD pattern of synthesized sample was shown in Fig.2.2.2. The XRD patterns of synthesized powders with it the characteristics pattern of HAP but not with much resolution and intensity. The broad peak around at (0 0 2), (2 1 1) and (2 0 2) indicates that the crystallites are very small in nature with much atomic oscillation. Fig. 2.2.2 shows that the collected XRD data was perfectly matched with standard ASTM data (JCPDS no 09-0432) and broaden diffraction peaks are 2θ=25.8, 31.7, 32.1 and 32.9 are assigned to the (002), (211), (112) and (300) miller’s indices reflection planes and calculated crystallite size was very small from 25-60 nm.
The FT-IR spectrum of the as prepared HAP was shown in Fig. 2.2.3. The peak at 3572.66 is due to the O-H stretch of water and HAP. The O-H groups are hydrogen bonded. The broad envelop at 1033 cm$^{-1}$ is due to P-O asymmetric stretching of $PO_4^{3-}$. The stretching and bending modes of $PO_4^{3-}$ appeared at 603.72 and 565.14 cm$^{-1}$. From the IR analysis, the precipitated powders were proved to be hydroxyapatite.

Fig.2.2.4 shows the SEM micrographs of HAP powders synthesized from the solution with pH 11. The as prepared pure HAP shows the uniform rod shape nano particles in the range of 30-60 nm. These pores are beneficial for the circulation of the physiological fluid throughout the coatings when it is used as biomaterials (Ruban Kumar et al; 2010).
Fig. 2.2.2 XRD patterns of synthesized HAP at 75°C
Fig. 2.2.3 FT-IR spectrum of as-prepared HAP
Fig. 2.2.4 SEM micrographs of as prepared HAP
Fig. 2.2.5 HRTEM micrograph of as prepared HAP
The High Resolution Transmission Electron Microscope (HRTEM micrographs of synthesized HAP are shown in Fig. 2.2.5 All HRTEM images show relatively uniform particles with a rod like shape. The length and width of the rods were in the range of 30-65 nm and 15-25 nm respectively.

2.2.4 CONCLUSION

Sol-gel technique, an elective method for the preparation of highly pure powder due to the possibility of a strict control of the process parameters helped to synthesis HAP in the range of 30-65 nm with agglomeration to certain extent. In this simple, low cost effect and also nanoparticle produced large scale in the coating purpose.

2.3. ULTRASONIC IRRADIATION TECHNIQUE

2.3.1 INTRODUCTION

Skeletal system consists of bone or osseous material comprising of a matrix hardened by deposited calcium phosphate and other minerals. Growing concern among aging population was bone disorder such as osteoporosis which was associated with bone loss and development of bone brittleness and fracture. Treatment of bone tumors and similar defects bone imitants were required to repair / replace the infected bone tissue and for the treatment of fractures (Fisher et al; 2002). Inquest into nanostructures and nano synthetic methods was paved way for the formation of different nano forms of HAP with many properties such as different shapes, rods, tubes, plates and spheres (Ramay et al; 2004, Fathi et al; 2008, Zhongli Shi et al; 2009).

Ardent efforts of researchers lead to the development of new methods for the synthesis of HAP including solid-state reaction, precipitation, Sol-gel, hydrothermal, electrophoretic deposition, microwave irradiation etc... A characteristic of the product depends on the method of synthesis. Depending upon the technique, materials with different morphology, stoichiometry, and level of crystallinity was obtained (Dong Seok Seo et al; 2008, Mamoru Aizawa et al; 2006). Ultrasonic has unique chemical effects, which is derived primarily from acoustic cavitation (Suslick S K; 1990). The aim this work was HAP nano particle synthesized by co-precipitation method assisted with
ultrasonic irradiation technique using following precursors - calcium nitrate tetrahydrate and di-ammonium hydrogen phosphate in the presence of aqueous 5% polyethylene glycol (PEG 600) under three pH (7, 9 & 11).

2.3.2 MATERIALS AND METHODS

Ca(NO$_3$)$_2$.4H$_2$O (99% pure), (NH$_4$)$_2$HPO$_4$ (99% pure) and ammonia solution, polyethylene glycol (PEG 600) were procured from Merck (Germany), and used without any further purification. HAP was synthesized under three different pH conditions (7, 9 and 11). Initially to start with, the HAP prepared for neutral pH, with 1M of Ca(NO$_3$)$_2$.4H$_2$O and 5% concentration of PEG (600) dissolved in 100ml of de-ionized water and this solution was stirred with the help of magnetic stirrer at 400 rpm for 8 hours to ensure proper molecular interaction. This mixture was transformed into heavy duty beaker and place inside ultrasonic horn bath (E-Chrom ultrasonic horn, operated 22 kHz frequency) at 85% amplitude with pulse duration of 10sec ON and 2sec OFF, for 30min, for six intervals of 5min duration each and was maintained at an elevated temperature of 70°C. As the solution reaches the said temperature 100ml of 0.6mole (NH$_4$)$_2$HPO$_4$ solution was added drop wise to the above mixture and the pH of the overall solution was maintained neutral (pH 7) until a slurry was obtained, the obtained slurry was allowed to settle for a week in closed beakers at room temperature. The obtained sediment was washed with double distilled water and ethanol of HPLC grade twice to remove nitrate ions and other organic impurities, and dried in a hot air oven at 80°C for 10hrs in order to obtain a fine powder nature. This procedure was followed for the preparation of nano size HAP at other two pH.

Powder X-ray diffraction results were carried out by BrukerD8 advanced diffractometer using source 2.2kW Cu anode, ceramic X-ray tube with CuK$_\alpha$ radiation wavelength (λ=1.5418Å). The 2θ range was from 20-55° in steps 0.02° and count time 0.2S. The XRD spectrum due to different phases compared to ASTM standards and the crystallite size (Xs) was determined by Debye-Scherrer formula. Fourier transform infrared (FTIR) spectroscopy was recorded by the KBr pellet technique using SHIMADZU IRAFFINITY spectrometer for the range 400-4000cm$^{-1}$ was used to
confirm the functional groups. A morphological study of the obtained HAP nano particle was analyzed by Quanta 200 FEG scanning electron microscope (SEM).

2.3.3 RESULTS AND DISCUSSION

Fig.2.3.1 shows the XRD pattern of HAP synthesized at three different pH under the influence of 5% PEG (600) acting as organic modifier. It was evident from Fig. 2.3.1 (a) which shows the diffraction peak pertaining to HAP synthesized at 7pH shows three crystalline phases pertaining to OCP at 26.5° in (122) plane, DCP at 29.5° in (113) plane and HAP at 31.7° in (211) plane. From Fig. 2.3.1(b, c) corresponding to pH 9 and 11 respectively, it was apparent that the peaks pertaining to OCP and DCP disappear completely indicating the presence of pristine HAP, which was in accordance with the JCPDS data (no.09-0432). The 2θ values in Fig. 2.3.1 (b, c) at 23.0°, 25.9°, 28.9° and 31.7°, 32.9°, 34.1° corresponds to miller indices planes (111), (002), (210), and (211), (300), (202) respectively. The said peaks were found to possess low intensity in Fig. 2.3.1(a), which could be attributed to the deficiency of calcium and higher crystalline size. The high intensity peaks appearing in 25° to 35° as shown in Fig. 2.3.1 (b, c). XRD spectrum shows that the crystallite size of the as-synthesized HAP was below 40 nm.

FT-IR spectra of the as-synthesized HAP powders in the presence of 5% PEG (600) with various pH condition as shown in Fig. 2.3.2 (a-c). The peaks appearing in the range above 3500cm\(^{-1}\) and 634cm\(^{-1}\) indicate (O-H) Hydroxyl functional groups and the absorption peak at 1030cm\(^{-1}\) indicate PO\(_4\)^\(^{3-}\) while 603 and 563cm\(^{-1}\) peaks correspond to (P-O) bending modes of vibration and broaden peaks appeared in above 3100 cm\(^{-1}\), which was clearly indicated HAP particle absorbed water molecule. The role of pH and the organic modifier could not be predicted from FTIR spectra.

The surface morphology of synthesized HAP at different pH conditions are shown in Fig. 2.3.3 (a-c). It is evident from the micrographs that the morphological appearance for the HAP nanoparticles were of rod shaped, less agglomerated when compared with higher pH 9 and 11, which show spherical morphology and agglomerated.
Figure 2.3.1 XRD pattern of HAP powder synthesized different pH values (a) pH7, (b) pH 9 and (c) pH11
Fig. 2.3.2 FTIR spectrum of synthesized HAP under various pH value, (a) pH7, (b) pH9, (c) pH11
Fig. 2.3.3 SEM micrographs of as-synthesized HAP at different pH condition, (a) pH 7, (b) pH 9 and (c) pH 11
Ultrasonic irradiation creates cavitation effects in an aqueous medium stimulating chemical reactivity producing heterogeneous reactions between Ca\(^{2+}\) and PO\(_4^{3-}\). This radiation process was very effective but cannot play any vital role in the synthesis of HAP in neutral pH. In this case, the reaction medium was inactive and Ca\(^{2+}\), PO\(_4^{3-}\) ions releasing rate was slow from the precursor solution to form calcium deficient hydroxyapatite, which was less agglomerated.

PEG-OH molecule easily attracts Ca\(^{2+}\) ions from the calcium precursor solution to form the bond of PEG-O-Ca\(_2\)-O-PEG (Kalita et al; 2007). PEG mixed Ca\(^{2+}\) ions were attracted by very fast PO\(_4^{3-}\) ions in pH 9 and 11 to form pure and agglomerated small crystallite size HAP particles with spherical morphology having some pores between the particles.

2.3.4 CONCLUSION

The HAP nanoparticle synthesized successfully in the presence of 5%PEG by coprecipitation method assisted with ultrasonic irradiation technique. At neutral pH, the reaction medium was inactive and the slow interaction of Ca\(^{2+}\), PO\(_4^{3-}\) ions to form calcium deficient HAP. For pH 9, interaction between ions Ca\(^{2+}\) and PO\(_4^{3-}\) was very fast because reaction medium was very active producing pure and agglomerated HAP nanoparticle. XRD results perfectly agreed with standard JCPDS data.

From this observation,

(i) Ultrasonic irradiation method was very effective in particle size reduction

(ii) pH value 9 is suitable for HAP nanoparticle synthesis with Ca/P ratio 1.67, and

(iii) Both ultrasonic irradiation process and pH values play a vital role in the formation of phase pure HAP nano particles.
2.4 CO-PRECIPITATION TECHNIQUE

2.4.1 INTRODUCTION

When compared to other methods co-precipitation synthesis has its own importance. The co-precipitation method is a versatile and economic route for the synthesis of homogeneous and high pure organic free HAP powders (Zhigang Liu et al; 2014). Advantage of this route is, it is quite simple and fast, easy to control the particle size and composition and various options in the surface modification of HAP particle and obtaining the total homogeneity (Mobasherpour et al; 2007).

2.4.2 MATERIALS AND METHODS

Ca(NO$_3$)$_2$.4H$_2$O and (NH$_4$)$_2$HPO$_4$ were used as starting material to provide Ca and P sources. The above chemicals were prepared by dissolving them into deionized (Milli-Q) water. The pure hydroxyapatite was prepared by mixing 1M Ca(NO$_3$)$_2$.4H$_2$O and 0.6M (NH$_4$)$_2$HPO$_4$. The pH was maintained at 10 by titrating with NH$_4$OH. A typical procedure is as follows: 1M of Ca(NO$_3$)$_2$.4H$_2$O was mixed with 0.6 M of (NH$_4$)$_2$HPO$_4$ solution with vigorous stirring. Using NH$_4$OH the pH was adjusted to10. The pure HAP solution was stirred for 3h and the pH was maintained at 10. The resultant precipitate was put into a refluxing system and this precipitate was refluxed 2 h with 85ºC. After cooling to room temperature, the precipitates were centrifuged, washed with deionized water and dried in vacuum at 80°C for 2 hrs (Zhigang Liu et al; 2014).

The X-ray powder diffraction patterns were recorded using D8 advanced BRUKER diffractometer with Cu Kα radiation (λ=0.15405 nm). The patterns due to different phases were compared with the ASTM standards. The samples were further characterized by FT-IR spectroscopy in the range of 400-4000cm$^{-1}$ using Perkin Elmer RXI FT-IR spectrometer by KBr pellet technique. The surface morphology of the samples was observed by JEOL- JSM-6510 scanning electron microscope. Transmission electron microscopy (TEM) images were taken from Philips, CM200, and operating voltage20-200kv. It gives a lattice resolution of 0.5nm and a point to point resolution of 0.2nm and also investigated the in-vitro anti-proliferative and hemolytic activity of HAP.
2.4.3 RESULTS AND DISCUSSIONS

The crystal structure of the products prepared from the solution with pH above 10 under co-precipitation route was examined by XRD analysis. All diffraction peaks can be indexed as the hexagonal HAP which is shown in Fig. 2.4.1. The XRD patterns of as-prepared sample shows diffraction peaks in good agreement with the ASTM data [JCPDS No. 09-0432]. No characteristic peaks of impurities, such as calcium hydroxide and calcium phosphates were observed, meaning that phase pure HAP was prepared under the present experimental conditions. The pattern shows broaden peaks particularly in the planes (0 0 2), (2 1 1), (1 1 2) and (3 0 0) reveals that the particles size are very small in the range of 30-50 nm and also uniform. The diffraction peaks are high and narrow, implying that the HAP crystallizes well.

The FTIR spectrum of the obtained HAP was shown in Fig. 2.4.2. This FTIR spectrum indicates that the small characterized peaks 3572 and 632 cm$^{-1}$ were assigned to hydroxyl (O-H) group. PO$_4^{3-}$ stretching vibration mode appears in 1093, 1033 and 962 cm$^{-1}$. While other two peaks 603 and 565 cm$^{-1}$ were assigned to the bending modes of vibration. Wave number 1714 indicates that strong intensity of (C-O) stretching vibration modes.

The FT-Raman spectrum of pure HAP was shown in Fig.2.4.3. This spectrum was recorded in the frequency range from 200-3800cm$^{-1}$. FT-Raman spectroscopy gives more information about the phosphate vibration modes. The sharp peaks 961cm$^{-1}$ and 1061cm$^{-1}$ were assigned to phosphate groups and especially, 1061cm$^{-1}$ peaks was appearing only in well crystallize HAP. The lower intensities bands were observed in the frequency 430 cm$^{-1}$, 591cm$^{-1}$. Fig. 2.4.4 shows different magnification of the SEM micrographs of HAP powders synthesized from the co-precipitation method. The as prepared pure HAP particles appear with the definite shape and non-uniform size rod like morphology with 35 to 65 nm respectively. The particles are agglomerated with each other leaving pores in the range of nano meters. The formations of pores are beneficial, as
Fig. 2.4.1 XRD patterns of synthesized HAP by co-precipitation method
Fig. 2.4.2 FTIR spectrum of synthesized HAP by co-precipitation method
Fig. 2.4.3 FT-Raman spectrum of synthesized HAP by co-precipitation method
Fig. 2.4.4 SEM micrograph of synthesized HAP by co-precipitation method
they would permit the circulation of the physiological fluid throughout the coatings when it is used as biomaterial. TEM images of the particles are shown in Fig. 2.4.5. TEM images reveal the presence of HAP in the range of 35-65 nm in agglomerated state. The multi-spot rings of the selected area diffraction (SAD) pattern for the HAP sample indicated nano crystalline HAP. Most of the nano-sized HAP particles tend to agglomerate although the particle dispersed ethanol solution was vibrated for 15 min in an ultrasonic water bath for the homogenization of the samples. The SAD patterns as shown in Fig. 2.4.6. The well-resolved lattice fringes confirm the high crystallinity of the as-prepared HAP. The distance (0.28nm) between the adjacent lattice fringes agrees well with the $d$ spacing of the literature value.

2.4.4 TEST FOR ANTI-PROLIFERATIVE ACTIVITY

2.4.4.1 MTT ASSAY

The anti-cancer activity of HAP was confirmed by MTT assay (1). A549 cells were seeded in 96 well plates using DMEM supplemented with 5% FBS. After reaching confluence, the cells were treated with varying concentrations (100-1000 μg/ml) of HAP and incubated for 24 h. After incubation 20 μl of MTT was added to the reaction and incubated for an additional 3 h at 37 °C in 5% CO$_2$ incubator. The supernatant was removed and 150 μl of MTT solvent was added to dissolve the formed formazan crystals. The purple formazan developed was measured at 570 nm using a spectrophotometer. The number of viable cells was proportional to the extent of formazan production. The cytotoxic effect of HAP in A549 cells was deduced through the production of a dose-response curve as shown in Fig. 2.4.7. The effect of various concentrations of HAP against A549 lung cancer cells was observed under the microscope. The morphology changes with various concentrations of HAP under phase contrast microscope were represented in Fig. 2.4.8.
Fig. 2.4.5 TEM micrograph of synthesized HAP by co-precipitation method
Fig. 2.4.6 Selected area diffraction (SAD) pattern of synthesized HAP by co-precipitation method
Fig. 2.4.7 Cytotoxic effect of a dose-response curve of as-synthesized HAP in A549 cells
Fig. 2.4.8 Morphological changes with various concentrations of HAP under phase contrast microscope
Fig. 2.4.9 *in vitro* hemolytic activity of HAP
2.4.4.2 HEMOLYTIC ACTIVITY

Hemolytic assay was carried out according to Bulmus et al (Bulmus et al; 2003). Fresh human red blood cells were washed thrice with 150 mmole of NaCl (2500 rpm for 10 min). The separated serum was suspended in 100 mmole of sodium phosphate buffer. Different concentrations of HAP, viz 50 μg, 100 μg, 150 μg and 200 μg were mixed with 200 μl of RBC solution and the reaction volumes were made up to 1 ml with sodium phosphate buffer. The reaction mixture was incubated at 37°C for 1 h and then centrifuged at 2500 rpm for 15 min. Optical density of the supernatant was measured at 541nm, using sodium phosphate buffer as blank. Deionized water was used as a positive control. The experiment was done in the duplicates and the mean was calculated as in Fig. 2.4.9.

\[
\text{Hemolysis activity(%) = } \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of positive control}} \times 100
\]

2.4.5 CONCLUSION

HAP nanoparticles were successfully synthesized by co-precipitation method. SEM and TEM micrograph shows that confirm the formation of synthesized HAP particles in nano scale level and FT-IR spectrum confirms the functional groups. The anti-proliferative and hemolytic activity of HAP suggests that, it was potential material for cancer treatment. This kind of well crystallized HAP was used in various biomedical applications such as bone cavity fillings, replacement of joints and this HAP also used as a drug delivery.
2.5 SYNTHESIS, CHARACTERIZATION, IN-VITRO ANTI-PROLIFERATIVE ACTIVITY OF FLUORAPATITE

2.5.1 INTRODUCTION

A traditionally used calcium phosphate ceramics widely used in the field of drug delivery carriers, catalyst carriers, adsorbents and acid base catalysis (Uddin et al; 2010, Telalovic et al; 2010, Akazawa et al; 1997, Zahouily et al; 2003). Fluorapatite (FA) was an alternative material for apatite derivative material due to its low solubility in organic solvents as well as water, higher acid resistance excellent biocompatibility and higher thermal stability (melting point of FA was 1650ºC) (Lugscheider et al; 1994, Zhao et al; 2007). The tooth enamel consists of apatite minerals containing 0.04wt.% to 0.07wt.% of fluorine. Moreover, microelement of fluorine was necessary for the normal dental and bone formation in the human body (Turner et al; 1993). In vitro test result demonstrated that the FA was biocompatible and controlled release of fluorine ions, these kinds of releasing fluorine ions increases of mineralization, bone density and prevalence of dental caries (Chen et al; 2006). FA was used to prepare the fluorescent lamps, laser host for neodymium and praseodymium ions, humidity sensors, water purification (Zhang et al; 1999, Zhang X X et al; 1994, Sardar et al; 2000, Nagai et al; 1998, Chen et al; 2008).

If the OH⁻ ions in HA was completely substituted by F⁻, fluorapatite (Ca₁₀(PO₄)₆F₂) was formed and this kind of fluorine substituted apatite was increase the chemical stability, mechanical stability, and a bone cell proliferation (Barinov et al; 2004).

In this section, FA nano particle synthesized by co-precipitation method assisted with ultrasonic irradiation technique. Confirmations of morphology and particle size carried out using scanning electron microscope and transmission electron microscope respectively are presented and also investigate the in vitro anti-proliferative and hemolytic activity.

2.5.2 MATERIALS AND METHODS

Ca(NO₃)₂.4H₂O (99% pure), (NH₄)₂HPO₄ (99% pure) and ammonium fluoride, ammonia solution were procured from Merck (Germany), and used without any further
purification. Initially, start with 5M of Ca(NO$_3$)$_2$.4H$_2$O dissolved in 100ml of de-ionized water and this solution was stirred with the help of magnetic stirrer at 600 rpm for 8 hours and then calcium precursor solution was transformed into heavy duty beaker and placed inside ultrasonic horn bath (E-Chrom ultrasonic horn, operated 22 kHz frequency) at 85% amplitude with pulse duration of 10S ON and 2S OFF, for 30 min, for six intervals of 5 min duration each and was maintained at an elevated temperature of 70°C. As the solution reaches the said temperature 100ml of 3mole (NH$_4$)$_2$HPO$_4$ solution and 1 mole ammonium fluoride solution was added drop wise to the above calcium precursor solution and the pH of the overall solution was maintained 11 (by using ammonia solution) until a slurry was obtained, the obtained slurry was allowed to settle for a week in closed beakers at room temperature. The obtained sediment was washed with double distilled water and ethanol of HPLC grade twice to remove nitrate ions and other organic impurities, and dried in a hot air oven at 120°C for 10 hrs in order to obtain a fine powder nature. Ultrasonic irradiation processor specifications are given in table 2.5.1.

<table>
<thead>
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<th>Table 2.5.1 Ultrasonic irradiation processor specifications</th>
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<td>Setup</td>
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<td>Rate output power</td>
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<td>Diameter of titanium tip horn</td>
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<td>Surface area of ultrasound irradiating face</td>
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Phase analysis were carried out by BrukerD8 advanced X-ray diffractometer using source 2.2kW Cu anode, ceramic X-ray tube with CuK$\alpha$ radiation wavelength ($\lambda$=1.5418Å$^\circ$). The 2θ range was from 05-55° in steps 0.02° and count time 0.2S. The diffraction pattern of FA was compared to ASTM standards and the crystallite size (Xs) was determined by using Debye-Scherrer formula. The functional groups of as-synthesized FA powders were analyzed by Fourier transform infrared (FTIR)
spectroscopy was recorded by the KBr pellet technique using SHIMADZU IRAFFINITY spectrometer. One milligram of FA powder was mixed with 100mg spectroscopic grade KBr by hand-milling the FA powder in an agate mortar and the transmission spectrum was recorded in the range 4000 to 400cm$^{-1}$.

A morphological study of the thus obtained FA nano particle was analyzed by scanning electron microscope 3.0kV and TEM (Philips, CM200, and operating voltage20-200kv). From the TEM analysis FA nano particle was prepared by solution-drop method. A small amount of FA powdered was added into a 10 ml methanol solution, and this ethanol solution dispersed in an ultrasonic water bath for 10 min. A drop of the dispersed solution was dropped onto the carbon coated copper grid using small-tipped transferring pipette. After vaporization of ethanol mixed FA solution and this FA material deposited on the carbon film. This FA coated copper grid was ready for the TEM observation and also investigated the in-vitro anti-proliferative and hemolytic activity of FA.

2.5.3 RESULTS AND DISCUSSIONS

The FTIR spectrum of as prepared FA is shown in Fig. 2.5.1. It shows the vibrational bands corresponding to the phosphate bands at 561,601, 964, 1026, and 1093. The bands at 964cm$^{-1}$ was corresponds to ($\gamma_1$) vibration peaks and absorption peaks occurred in 1026 and1093cm$^{-1}$ were derived from the asymmetric stretching of ($\gamma_3$) (PO$_4$)$_3$, and at 561 and 601cm$^{-1}$ were corresponding to the bending modes of ($\gamma_4$) (PO$_4$)$_3$vibration, respectively.

The FT-Raman spectrum of as prepared FA was shown in Fig. 2.5.2. This spectrum was carried out the room temperature with performed Bruker RFS27 instrument using Nd: YAG laser with an excitation wavelength 1064 nm and spectral resolution 1cm$^{-1}$. This spectrum was recorded in the frequency range from 200-3800cm$^{-1}$. FT-Raman spectroscopy gives clear information about the phosphate vibration bonds. The sharp peaks ($\gamma_1$) 961cm$^{-1}$and ($\gamma_3$) 1061cm$^{-1}$ were corresponded to apatite phosphate groups and especially, 1061cm$^{-1}$ peaks was appearing only in well crystallize FA. The lower intensities bands were observed in the frequency ($\gamma_2$) 430cm$^{-1}$, ($\gamma_4$) 591cm$^{-1}$. 

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Fig. 2.5.1 FTIR spectrum of synthesized FA
Fig. 2.5.2 FT-Raman spectrum of synthesized FA
Figure 2.5.3 XRD spectrum of synthesized FA
The crystal structure of the FA prepared from the solution with pH above 10 under ultrasonic irradiation route was examined by XRD analysis. All diffraction peaks can be indexed as the hexagonal FA which is shown in Fig. 2.5.3. The XRD patterns of as-prepared sample shows diffraction peaks in good agreement with the ASTM data [JCPDS No. 15-0876]. No characteristic peaks of impurities, such as calcium hydroxide and calcium phosphates were observed, meaning that phase pure FA was prepared under the present experimental conditions. The pattern shows broaden peaks particularly in the planes (0 0 2), (2 1 1) and (3 0 0) reveals that the particles size are very small in the range of 19-35 nm and also uniform. The diffraction peaks are high and narrow, implying that the FA crystallizes well.

Fig. 2.5.4 shows different magnification of the SEM micrographs of FA nano particle synthesized from the ultrasonic irradiation method. The as prepared pure FA particle appears with the definite shape and uniform rod like shape with 19 to 35 nm respectively. The particles are agglomerated with each other leaving pores in the range of nano meters. The formations of pores are beneficial, as they would permit the circulation of the physiological fluid throughout the coatings when it is used as biomaterial.

TEM images of the particles are shown in Fig. 2.5.5. TEM images reveal the presence of HAP in the range of 19-35 nm in agglomerated state. The multi-spot rings of the selected area diffraction (SAD) pattern for the FA sample indicated nano crystalline FA. Most of the nano-sized FA particles tend to agglomerate although the particle dispersed ethanol solution was vibrated for 15 min in an ultrasonic water bath for the homogenization of the samples. The SAD patterns as shown in Fig.2.5.6. The well-resolved lattice fringes confirm the high crystallinity of the as-prepared FA. The distance between the adjacent lattice fringes agrees well with the \( d \) spacing of the literature value.
Fig. 2.5.4 SEM micrograph of synthesized FA
Fig. 2.5.5 TEM micrograph of synthesized FA
Fig. 2.5.6 Selected area diffraction (SAD) pattern of synthesized FA
Fig. 2.5.7 Cytotoxic effect of a dose-response curve of as-synthesized FA in A549 cells
Fig. 2.5.8. Morphological changes with various concentrations of FA powders under phase contrast microscope
Fig. 2.5.9 *in vitro* hemolytic activity of FA
2.5.4 TEST FOR ANTI-PROLIFERATIVE ACTIVITY

2.5.4.1 MTT ASSAY

The anti-cancer activity of FA was confirmed by MTT assay (1). A549 cells were seeded in 96 well plates using DMEM supplemented with 5% FBS. After reaching confluence, the cells were treated with varying concentrations (100-1000 μg/ml) of FA and incubated for 24 h. After incubation 20 μl of MTT was added to the reaction and incubated for an additional 3 h at 37 ºC in 5% CO₂ incubator. The supernatant was removed and 150 μl of MTT solvent was added to dissolve the formed formazan crystals. The purple formazan developed was measured at 570 nm using a spectrophotometer. The number of viable cells was proportional to the extent of formazan production. The cytotoxic effect of FA in A549 cells was deduced through the production of a dose-response curve as shown in Fig. 2.5.7. The effect of various concentrations of FA against A549 lung cancer cells was observed under the microscope. The morphology changes with various concentrations of FA under phase contrast microscope were represented in Fig. 2.5.8.

2.5.4.2 HEMOLYTIC ACTIVITY

Hemolytic assay was carried out according to Bulmus et al (Bulmus et al; 2003). Fresh human red blood cells were washed thrice with 150 mmole of NaCl (2500 rpm for 10 min). The separated serum was suspended in 100 mille mole of sodium phosphate buffer. Different concentrations of FA, viz 50 μg, 100 μg, 150 μg and 200 μg were mixed with 200 μl of RBC solution and the reaction volumes were made up to 1 ml with sodium phosphate buffer. The reaction mixture was incubated at 37°C for 1 h and then centrifuged at 2500 rpm for 15 min. Optical density of the supernatant was measured at 541nm, using sodium phosphate buffer as blank. Deionized water was used as a positive control. The experiment was done in the duplicates and the mean was calculated as in Fig. 2.5.9.

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\text{Hemolysis activity(\%)} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of positive control}} \times 100
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2.5.5 CONCLUSION

We have successfully developed a new approach to preparing phase pure, hexagonal, and rod shape nano FA powders at a low temperature. More importantly, as-synthesized FA nano rod shape particles possess the anti-proliferative and hemolytic activity. In this ultrasonic irradiation process was very simple and aqueous based; gel formation was without using any external agents and nano FA was prepared without any need grinding process. XRD, FTIR, FT-Raman and SEM, TEM have been used to analysis the phase, crystallite size and morphology of as-synthesized FA particle. Above mention characterization results shows that synthesized FA was phase pure and rod like morphology with 19×32 nm. The ultrasonic irradiation method was very simple and inexpensive. Therefore, it was suggested that preparing pure, stoichiometry, nano crystalline FA needed for the large-scale productions of various biomaterial applications such as coating, drug delivery and catalyst carriers.