International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.9, No.03 pp 267-276, 2016

Assessment of Different Synthesis Route of Hydroxyapatite and Study of its Biocompatibility in Synthetic Body Fluids

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Abstract: Biomaterials have found its extensive application in various fields and are widely accepted due to its non-viable properties and biocompatibility. There are several types of biomaterials and hydroxyapatite (Hap) is one of the material which is easy to synthesize and is used rapidly in the field of medical sciences. In the present study, different methods like sonication, precipitation and sonication with precipitation method were employed in order to evaluate the preeminent method for the synthesis of Hap. The characterization of the material was carried out by Fourier transmission infra red spectroscopy (FT-IR), X-Ray diffraction (XRD) and Scanning electron microscopy (SEM). Characterization studies confirmed the feasibility of hydroxyapatite synthesis using all the methods employed. In this study it was found that sonication followed by precipitation was the best suited method for the synthesis of hydroxyapatite. In-vitro assessment for biocompatibility of synthesized Hap was done by using synthetic body fluids (SBF) solution. The morphology, surface characteristics as well as biocompatibility of the obtained Hap was analysed from SEM studies. It was observed that a formation of apatite layer took place on the surface of hydroxyapatite when kept in synthetic body fluids (SBF) solution due to its nature of bioactivity
Keywords: Biomaterials, biocompatibility, bioactivity, hydroxyapatite.

Introduction and Experimental

Bone transplant and its replacement is done using bone graft which includes auto grafts, allografts, and synthetic grafts. Now a days auto grafts are being used worldwide due to its outstanding biocompatibility, no immunogenic response and better osteogenic properties [1-4]. Problem lies in their availability and also they requires surgical procedure for reaping which results in pain, nerve damage and it also have potential risk of diseases like Human Immunodeficiency diseases. [5,6]. In case of allogenic grafts, bone grafts can be obtained easily but allogenic grafts may cause immunogenic response, microbiological infection and problem with incorporation [7-9].

Advancement in technology leads to development in implant materials which are non-corrosive and biocompatible in nature [3,10]. One such biomaterial which can be used as a replacement is hydroxyapatite due to its chemical and structural similarities with bone mineral. It is well-thought-out to be biocompatible with various body tissues and it also possess decent osteoconductivity and slow biodegradability. Slow biodegradation of hydroxyapatite will support in the formation of blood vessels which are essential for the establishment of bone tissues as it will consent the cells to colonise the implant material [11-16]. In physiological environment, hydroxyapatite is considered as the steady calcium phosphate compound. Synthetic

hydroxyapatite [Ca_{10} $(PO_4)_6$ $(OH)_2$] is a bio-ceramic which is porous and granulated in nature, has found its wide application in the field of biomedical, dentistry, orthopaedics, waste water and drinking water treatment [17-19]. Hydroxyapatite is used as scaffold in bone draft, granules or coating and as an alternative material for bone implants. Various techniques are now been employed for the formation of scaffolds which can be used in bone tissue engineering [20-22].

The above applications led to increase in the use of hydroxyapatite and increasing the curiosity of researchers, in the field of biomaterials. Improving the pore size and there in-vitro and in-vivo assessment is an important aspect in order to check the biocompatibility and biodegradation of the material synthesized. Various methods like sol-gel approach, multiple emulsion technique, electrodepositing techniques, precipitation, hydrothermal and self-assembly methods have been employed for the purpose of synthesis of hydroxyapatite with respect to different applications [23-27]. From all of the mentioned techniques, precipitation method has gained most popularity due to its simple procedure and cost effective synthesis [26, 28].

The present report illustrates the synthesis of Hap using chemical route method and its in-vitro assessment for biocompatibility and biodegradability. Synthetic body fluids (SBF) solution is used to evaluate the biocompatible properties of synthesized Hap. SBF possess similar chemical properties as of human plasma [29, 30]. Formation of apatite layer on the surface of Hap when in contact with SBF solution can be linked with the in-vivo conditions which would take place when Hap will be used as scaffolds for various applications. Three different techniques such as sonication, precipitation and sonication with precipitation were employed for the synthesis of hydroxyapatite. This has evolved the materials with different properties and there compound analysis was done by using FTIR, phase analysis by XRD and surface morphology is evaluated by SEM. The best suited method among the three were used to synthesize Hap in order to examine its biocompatibility and biodegradability of the material. Synthesis of Hydroxyapatite and characterization of the best suited method will provide an easy approach for hydroxyapatite synthesis and Hap formed using this approach will be in pure and crystalline in nature.

Experimental

Materials

Calcium Nitrate (Ca $(NO_3)_2$, Loba chemie) and potassium phosphate (KH_2PO_4, Fisher) Analytical grade, were weighted according to the stoichiometry required for the synthesis of hydroxyapatite (molar ratio of Ca/P=1:67). pH of the solution was maintained by Ammonia Solution (NH_4OH, Loba Chemie). Once the solutions were prepared, three different methods were employed in order to evaluate the best suited method for the synthesis of hydroxyapatite and to check the biocompatibility of the material synthesized. Tris-HCl buffer solution was prepared by adding and dissolving, 0.1M Tris-HCl solution in 100 ml of distilled water. The pH of the buffer was maintained at 7.4 with the help of 1M HCl solution. Prepared Tris-HCl buffer solution was then used in studying biodegradation of Hap.

Synthesis of Hydroxyapatite using conventional precipitation method:

The solution of 0.32M, 40 ml Ca $(NO_3)_2$ and 0.19M, 60 ml KH_2PO_4 was taken into beaker and burette respectively. pH of the reagents was maintained at 9-10 by adding NH_4OH. Using precipitation method, potassium phosphate was added drop wise into the solution of calcium nitrate placed on magnetic stirrer (REMI, 2MLH). Various parameters such as temperature, pH and speed was taken into consideration and was maintained at 70-85^oC, 9 pH and 600 to 1000 rpm respectively.

Synthesis of Hydroxyapatite using sonication method:

Sonication is employed for synthesis of Hydroxyapatite under controlled parameters such as temperature and pH. Solution of $Ca(NO_3)_2$ was sonicated for 1 hour at a frequency of 30-40 KHz before starting of synthesis process. KH_2PO_4 was added drop-wise into the solution of $Ca(NO_3)_2$ which was placed in sonicator (Citizen, CD-4820). pH of the reagents was maintained at 9-10, throughout the process so that proper reaction between the two solutions could take place. When the titration was about to complete, white color precipitates were observed which indicated the formation of Hydroxyapatite.

Synthesis of Hydroxyapatite using Precipitation with sonication method:

Due to the effect of sonication and magnetic stirring together, highly homogeneous and concentrated material will be obtained which will be of appropriate pore size and morphology. The solutions prepared were sonicated for 1 hour after pH of both the reagents was maintained at 9. After sonication, conventional precipitation method was employed for the synthesis of hydroxyapatite. Different parameters which were employed for three different methods are given in table 1.

Table 1: Different techniques and conditions for HAP synthesis.

Sample	Method employed	Parameters
Hap-1	Sonication method	30-40KHz, 1 hour
Hap-2	Precipitation method	600-1000 rpm, Temp. 70-85^{0}C
Hap-3	Sonication with Precipitation method	1st 30-40KHz, 1 hour + Magnetic Stirring, 600-1000 rpm Temp. 70-850C

Processing of solution:

Solution obtained after synthesis from above mentioned methods were kept for 24 hours ageing. Centrifugation was used to separate out the precipitates. Precipitates thus obtained in the form of gel were washed with deionized water and then they were dried at 110^{o}C for about 5-6 hours. Dried form of Hap was crushed properly into a white powdered product. The synthesized powder was exposed to 750-800^0 C for calcination in muffle furnace for 2 hours. It has been reported that calcination improves the mechanical properties of HAP.

Characterization:

In order to evaluate the product morphology, compound analysis and phase determination; scanning electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FT-IR, Perkin Elmer spectrum) and X-ray diffraction (XRD, X`Pert Powder Panalytical, CuKα radiations) were used respectively. The 2Θ angle range used was from 10º to 80º with the scan step time of 0.500 per sec.

Fabrication of pellets

Calcined hydroxyapatite powder were then given in the shape of pellets by applying pressure of 2 tons for 1 minute using palletizer. Every time before loading sample into the dye, it was cleaned properly so that sticking doesn't occur and pellets can be formed in an effective manner. The pellets obtained from calcined hydroxyapatite were utilized for in-vitro assessment of hydroxyapatite as well as test for its biocompatibility.

In-vitro studies

Preparation of Synthetic Body Fluid (SBF): Compositions of chemicals were added in an appropriate amount with sequence as listed in table 2.

Table 2: Chemical composition of SBF solution.

S.No	Reagent	Amount (g/l)
1	NaCl	6.54
2	$NaHCO_3$	2.26
3	KCl	0.37
4	$Na_2HPO_{4.2}H_2O$	0.17
5	$MgCl_2.6H_2O$	0.31
6	$CaCl_2.2H_2O$	0.36
7	Na_2SO_4	0.07
8	$(CH_2OH)_3CNH_2$	6.1

Patent pending. "Turkish Patent Institute," Turkey, Appl. No. 99-0037, 11 January 1999.

The chemicals listed in table 2 were dissolved in 700 ml of distilled water until were completely dissolved in water. Before the addition of 6^{th} reagent that is $CaCl_2.2H_2O$ nearby 15 ml of 1M HCl was added to avoid turbidity. After the addition of 8^{th} reagent temperature was maintained at 37^0 C in water bath while titrating with 1 M HCl until pH is set to 7.4. During the titration process distilled water was also added simultaneously to make up the volume to 1L (1000 ml). In the whole process up to 40 ml of 1M HCl was utilized for pH adjustment and preparation of SBF solution. The solution was then utilized for biocompatibility analysis and remaining solution was stored at 4^0C.

Test for in- vitro Biocompatibility

In-vitro studies of Hydroxyapatite was done by using synthetic body fluid (SBF) which was prepared in-vitro. The pallets fabricated were used for biocompatibility studies. The pellets were soaked in SBF solution for 14 days and the pH of SBF solution was maintained at 7.4. Change in the morphology of the sample soaked in SBF solution was observed by Scanning electron microscopy.

Results and discussion

Hydroxyapaytite was synthesized successfully by three different methods and were characterized by FTIR and XRD analysis in order to reveal which method is the method for the synthesis of pure hydroxyapatite.

FTIR

FTIR was used in order to characterize different compounds present in hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. FTIR spectrum of Hap synthesized using three different methods is shown in fig1. Spectra were taken in the range of 500 cm^{-1} to 4000 cm^{-1}. It has been reported that peaks obtained in the region of 3400-3600 cm^{-1} and 450-550 cm^{-1} determine the presence of –OH stretching bond in Hydroxyapatite [31-32] and of 560-640 cm^{-1}, 963 cm^{-1}, and 1000-1100 cm^{-1} represents the presence of stretching modes of P-O bond in Hydroxyapatite [33] . The FTIR pattern of Hap synthesized using different methods is shown in figure 1.

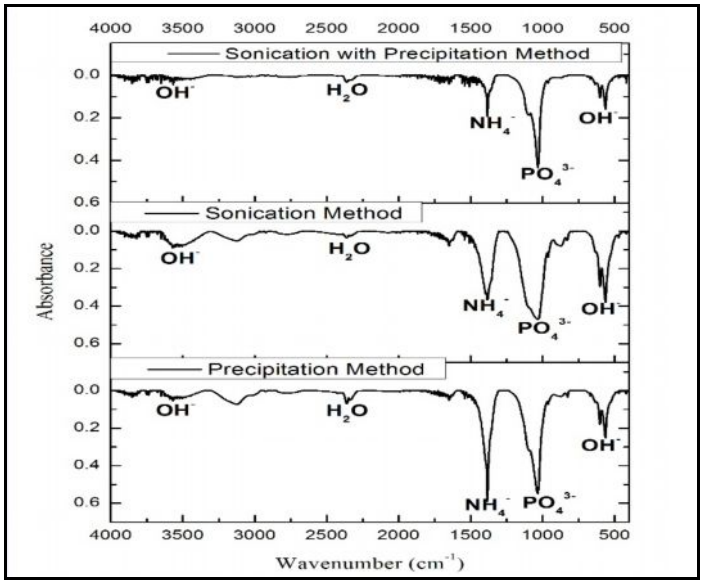

Fig.1 FTIR spectra of Hap synthesized from different methods.

FTIR spectrum shows all the specific peaks related to hydroxyapatite. FTIR band obtained at nearby 1000-1100 cm^{-1} is due to stretching of P-O bond [33]. The band obtained in the region of 1400 cm^{-1} is due to presence of NH_4^- group which was used to maintain pH during the reaction [34-35]. Most of the ammonia was removed with repeated washing but still there is a possibility of its remains which can be seen through the spectra. A very small peak of H_2O was obtained at 2400 cm^{-1} [36] Most of the physically adsorbed water was removed with drying and it can been seen from the figure above that the least peak of adsorbed water is obtained using sonication with precipitation method. The peak of NH_4^- decreased when sonication with precipitation method was used for synthesis of hydroxyapatite. The presence of P-O and O-H group almost confirms the formation of hydroxyapatite. Thus, from FTIR analysis it can be observed that out of different methods sonication with precipitation is the best method for the synthesis of hydroxyapatite.

XRD Analysis

The phase determination of Hap powder synthesized from three different methods were determined by X-ray diffraction analysis. XRD patterns of synthesized Hap from different method is shown in figure 2. For crystalline materials it is predicted that patterns obtained from XRD analysis are very sharp and peak broadening is related with crystal size [37].

All the peaks were matched with the standard JCPDS card number 09-0432. In sonication method the maximum peak intensity was observed at $2\theta = 32.2954^0$. Few peaks of tri-calcium phosphate was also observed which is due to the fact that, some of the calcium deca-phosphate had converted into tri-calcium phosphate which act as impurities. In precipitation method maximum intensity was obtained at an angle of $2\theta = 32.2954^0$. Other than Hap there are few phase of $CaCO_3$ which was observed when magnetic stirring method was employed for the synthesis of Hap.

According to the results obtained from XRD spectra it was seen that in case of Sonication with precipitation method the peak intensity was 741.2351 which is more if compared with sonication and precipitation method and angle at which intensity was obtained is at $2\theta = 31.7684^0$. More the intensity more is the sharpness of the peaks and hence the material is crystalline in nature. There was gradual decrease in peak intensity of $CaCO_3$ when hydroxyapatite was synthesized using sonication with precipitation method. This indicates the high purity of Hap synthesized using this method.

According to the standard from National Institute of Standard and Technology[(2)] and JCPDS card number 09-0432 it was seen that 2θ angle for HAP was obtained at 31.77^0 with maximum relative intensity (I_{rel}) = 100. The 2θ angle for sonication with precipitation method is approximately equal to the standard with highly pure and crystalline form of Hap.

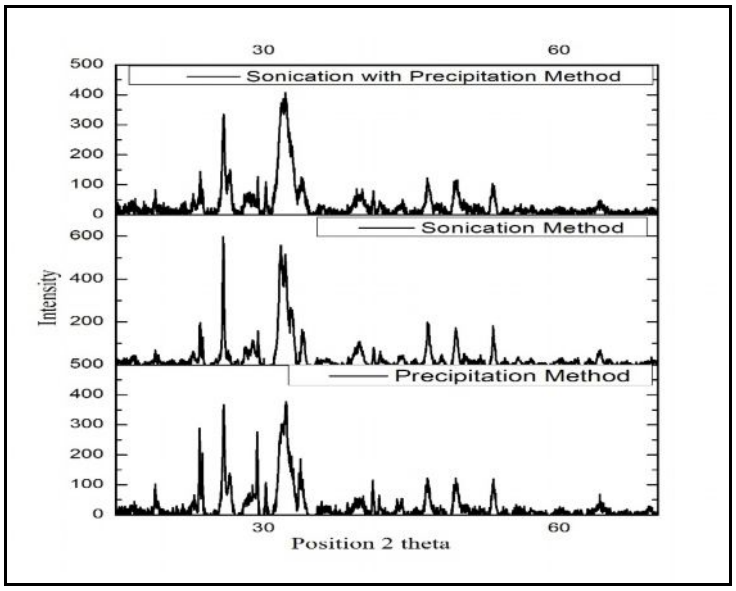

Fig.2 XRD pattern of Hap synthesized from different methods

Table3: Different 2 θ angle and related Peak Intensity of HAP synthesized from Different method

S.No.	Method for Synthesis	Peak intensity	Angle 2 θ of spectra obtained
1	Sonication Method.	601.759	32.2104
2	Magnetic Stirring Method.	611.795	32.2954
3	Sonication + Magnetic Stirring Method.	741.235	31.7684

The results obtained from FT-IR and XRD confirmed that best suited method for synthesis of Hap among the three is Sonication with Magnetic Stirring Method. In-vitro studies of Hap were carried out using the hydroxyapatite synthesized from best suited method.

Test for in- vitro Biocompatibility

The sample was under observation for 3 to 14 days and the properties of the solution such as pH was evaluated at regular interval. The layer of apatite was formed which increased with number of days as shown in figure 5-8. It was clearly assessed through SEM micrograph that there is a formation of apatite layer that is increased from day 3 to day 14. With increase in agglomerated particles it is the evident that there is formation of apatite on the surface of hydroxyapatite. Formation of apatite layer on the surface of Hap shows the biocompatibility of the material [38]. SEM micrograph of Control sample is shown in figure 4. Observation was taken after 3 days immersion of Hap pellets in the solution of synthetic body fluids. Formation of calcium precipitate can be clearly seen from the figure 5. Apatite is loosely adhered on the surface of hydroxyapatite [39]. Region marked in the figure 5b clearly depicts the settling of calcium ions, it also repesents that there is initation of the formation of apatite layer on the surface of Hap.

It was clearly seen from figure 6 that calcium ions which were deposited earlier had enticed phosphate ions which were present in SBF solution and formed a very thin layer of apatite after seven days immersion of hydroxyapatite in the solution of SBF. More clear results for the formation of apatite layer were attained from SEM micrograph shown in figure. 7 when Hap was immersed in SBF solution for ten days. Calcium rich and calcium deficient regions were easily observed from the micrograph. Due to the formation of calcium rich and calcium deficient regions, the formation of apatite layer occurs which was assessed from the results obtained after 14 days soaking of Hap in SBF solution. Under the layer of apatite there are particles of Hap which can be seen clearly through the SEM micrograph. A vibrant observation of the formation of apatite layer on the surface of synthesized hydroxyapatite is observed from the micrograph obtained after 14 days soaking of Hap in the synthetic body fluids solution. The properties of the SBF solution were evaluated at regular interval and pH was maintained at 7.4. Micrograph obtained after 14 days is shown below in figure 8. From the results obtained from

SEM it is clear that formation of apatite layer is taken place on the surface of Hap after the formation of Ca-rich and Ca-deficient regions which clearly depicts the biocompatibility of the material which was synthesized using Sonication with precipitation method. Small thread like structure obtained was not present in three days and not even seen in seven day immersion period. It was increased with time as seen through micrograph obtained for ten and fourteen days.

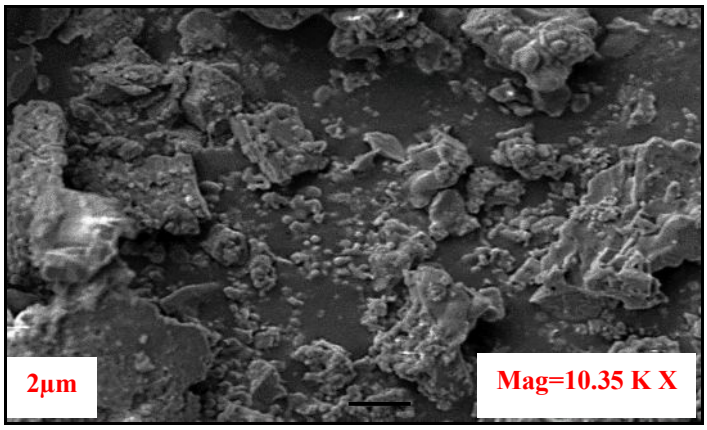

Fig.4: SEM micrograph of control sample

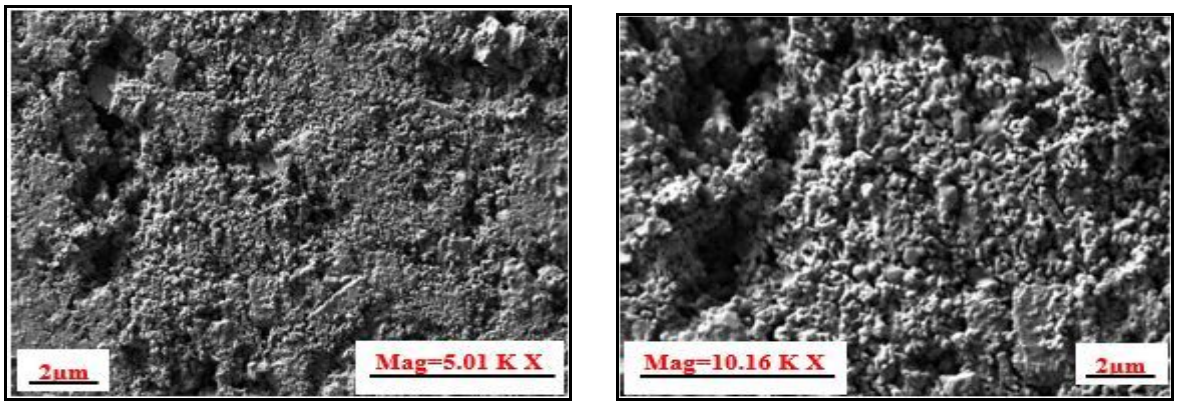

Fig5: Images by SEM at different magnification of the surface of HAP immersed for 3 days in SBF solution.

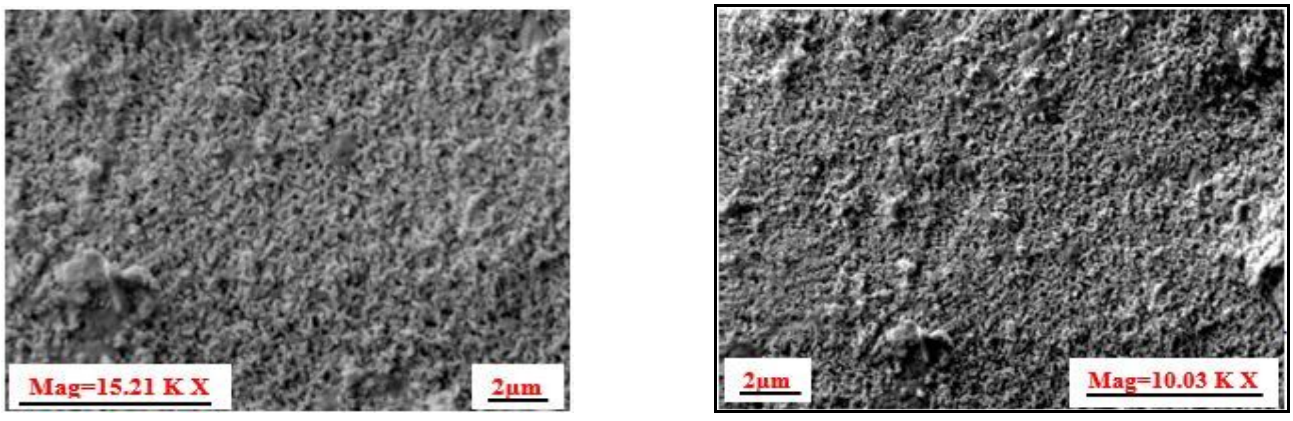

Fig6: SEM micrograph after seven days soaking in SBF at different magnification.

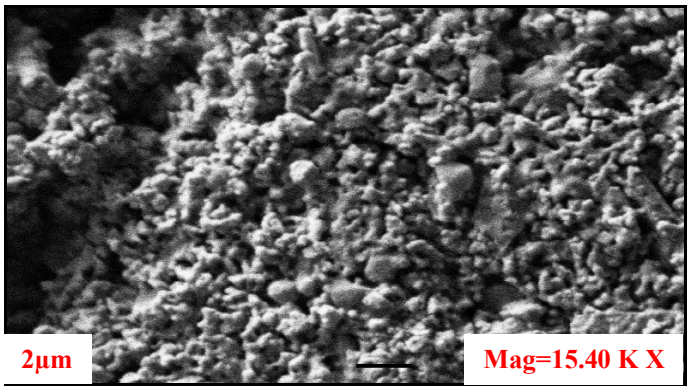

Fig. 7. (E); resolution at 15.40 K X, shown the Calcium-rich and Calcium- deficient regions.

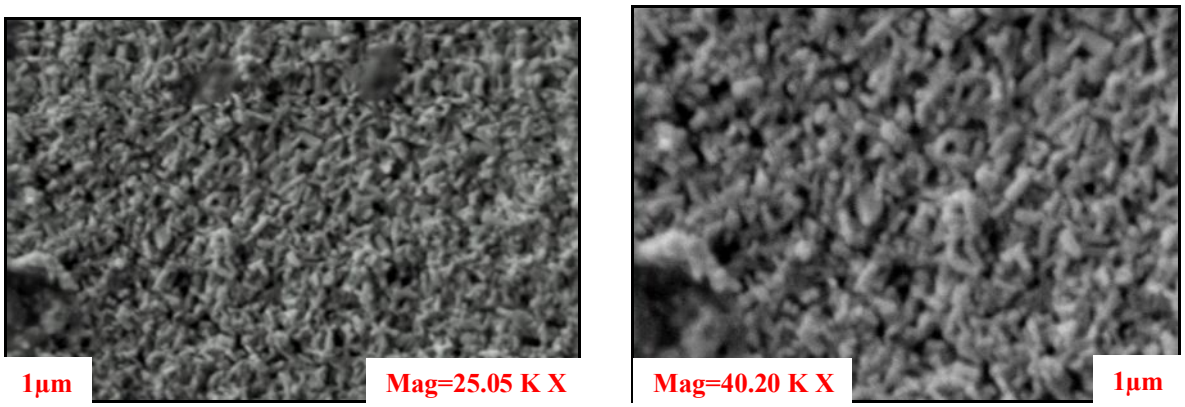

Fig8: SEM micrograph after fourteen days soaking in SBF at different magnification.

Mechanism of formation of Apatite layer on hydroxyapatite surface:

When surface of hydroxyapatite came directly in contact with SBF solution under normal condition, calcium ions start settling on the surface of Hap which are present in SBF solution resulting in the formation of Ca- rich region. The formed calcium rich region further attracts phosphate groups from SBF solution which results into the formation of Ca-deficient region [27]. Due to soaking of hydroxyapatite in SBF solution a layer of apatite is formed on the surface of Hap. A series of steps which took place when Hap was exposed to SBF solution is explained in figure 9.

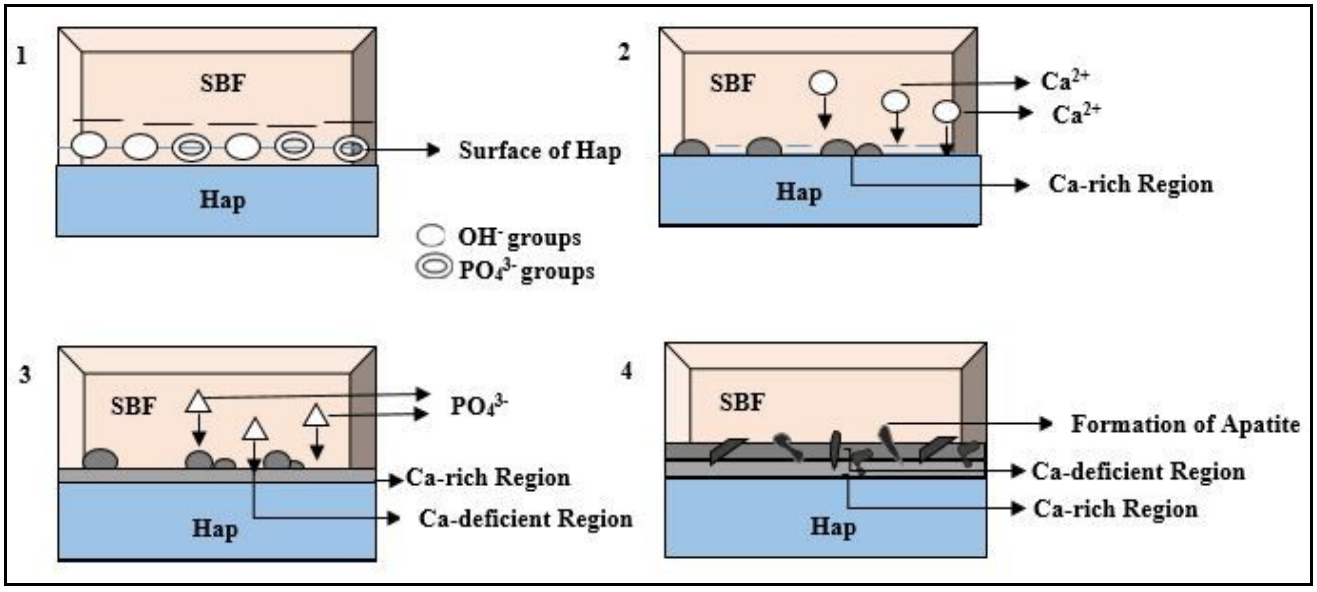

Fig.9 Formation of Apatite layer on the surface of hydroxyapatite.

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