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SYNTHESIS AND CHARACTERIZATION OF NANOHYDROXYAPATITE AND HYDROXYAPATITE/POLYCAPROLACTONE NANOCOMPOSITES

Rayasa RAMACHANDRA RAO* and Loganathan MARIAPPAN

Materials Science Division, CSIR - National Aerospace Laboratories, Old Airport Road, Kodihalli, P.B. No. 1779, Bangalore 560 017, INDIA (rrrao@nal.res.in)

Abstract:

Hydroxyapatite (HAp) - polycaprolactone (PCL) composite has found enhanced interest as scaffold material for bone tissue engineering application. The HAp phase is bioactive and provide the favorable environment for cell adhesion, proliferation, differentiation and cell conduction whereas the biodegradable PCL provide the required flexibility, mouldability and resorbability. Nanoparticles of HAp has been synthesized by precipitation method from $Ca(NO_3)_2.4H_2O$ and H_3PO_4 as precursors of Ca and P using tetrahydrofuran (THF) as the medium. The powder prepared was characterized for phase purity, functional group, particle size, surface area and morphology in comparison with the powder prepared using deionised water as the medium. By following the similar procedure of precipitation, nanoHAp was prepared in the PCL matrix with HAp to PCL ratio of 80:20 by wt % and HAp-PCL composite powder was filtered, dried at <50°C and characterized. The synthesized HAp and HAp-PCL composite show nano sized primary particles having X-ray pure hydroxyapatite phase. The HAp-PCL composite having homogeneous distribution of nanoHAp particles in PCL matrix could be a potential scaffold material for tissue engineering applications.

Keywords: Nano Hydroxyapatite; NanoHAp-PCL composite; In situ synthesis; Tissue engineering

1. Introduction

Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (HAp) a synthetic calcium phosphate has been proved as a bone regenerative material with its biocompatibility and bioactivity. HAp is being under consideration as one of the bone substitute material in orthopedic and dentistry over the last few decades. HAp has ability to form chemical bonding with surrounding tissues through formation of a microenvironment favorable for the nucleation and growth of biological apatite. HAp used in various forms (powder, coating, bulk or porous) has found to favor cell adhesion, differentiation and proliferation as well as osteoinduction and conduction. Further in nanometric scale it shows enhanced bioactivity and bone bonding phenomena. However, the exclusive use of HAp in tissue engineering applications is restricted by its limitations of brittle nature and relatively slow biodegradation [1,2].

Natural bone consists of about 70% inorganic phase made of micro crystals of HAp and 30% organic phase made of collagen matrix. The composite structure of bone responsible for higher mechanical properties as compared to synthetic HAp imposed the formation of composites of HAp with biocompatible and biodegradable polymers to result in a system morphologically and functionally similar to bone tissue. The composite is formulated to exhibit both the osteogenic characteristics of HAp and biodegradability of the polymer along with improved mechanical properties [3].

Synthetic polymers like polycaprolactone (PCL), polyglycolids (PGA), polylactides (PLA) and their co polymers are the most commonly used polymers in tissue engineering applications [3, 4]. PCL is biocompatible and biodegradable aliphatic polyester having low melting point and dissolves well in common organic solvents. PCL has been approved

by US Food and Drug Administration (FDA) for biomedical applications and has been under investigation for tissue engineering and drug delivery applications [3-7].

Different studies were carried out to fabricate HAp-PCL composite scaffolds with HAp concentration varying from 0 to 40% in PCL matrix [6, 7]. Various fabrication methods adopted include thermally induced phase separation [3], Solvent casting and particulate leaching [5.8], blending in melt form [9], computer controlled deposition or additive manufacturing processes [10, 11], gas foaming and microsphere sintering [12]. In these studies, the mechanical performance, biodegradability as well as in vitro and in vivo biological response of human bone cells were investigated. Overall results show that HAp-PCL scaffolds had better mechanical property, higher mineralization ability as well as better osteoblast interaction in terms of cell adhesion, proliferation and osteoconduction as compared to pure PCL scaffolds [6, 9-12]. PCL or HAp-PCL composite coatings were done on porous HAp, TCP or biphasic scaffolds and drug release studies were carried out by Kim *et al* [7] and Tarafdar and Bose [13]. The PCL coatings on HAp/TCP scaffolds resulted in controlled drug release for prolonged periods due to controlled degradation of PCL phase.

The above studies with an approach of mixing HAp powder from different sources with commercially available PCL to make the composite, had the drawbacks of variable grades of commercial HAp with varying crystallinity, purity, particle size and morphology. Further the physical mixing of the two components results in poor homogeneity in distribution of ceramic particles in polymer matrix due to which the improvement in mechanical properties was not attained as expected. In order to overcome these shortcomings, the new approach is to synthesis HAp directly in the presence of polymer dissolved in a solvent. Many papers are available for the synthesis of HAp in aqueous media [14, 15]. However, as PCL polymer is only soluble in organic solvent the HAp has to be synthesized in non-aqueous media. Literature available on this is very scanty.

Daiwon Choi *et al.* [16] synthesized HAp-PCL composites containing up to 34.5 wt% of single phase HAp through precipitation from calcium nitrate tetrahydrate and phosphoric acid in tetrahydrofuran (THF) as solvent and mixing with PCL solution in THF. HAp obtained are 1-10 µm sized agglomerated clusters of 20-25nm spherical crystals distributed homogenously in the crystalline matrix of PCL. Good adhesion between the HAp particles and PCL matrix through hydrogen bonding has been proposed by SEM and IR spectral analysis as well as by improvement in young's modulus of PCL by incorporating HAp particles.

On the contrary to the above solution mixing method, Choi and co-workers [17] prepared hybrid PCL-HAp nanocomposites containing 0-30 wt % of HAp by in-situ co-precipitation method through addition of H_3PO_4 to the solution containing PCL and $Ca(OH)_2$ in required proportions taken in THF solvent. The in-situ synthesized HAp had elongated nanocrystals dispersed uniformly in the PCL matrix without severe agglomeration. The composites show improved bioactivity in terms of osteoblast cell differentiation measured through alkaline phosphatase activity as compared to conventionally prepared PCL/HAp composite which is attributed to the nanoscale hybridization of the HAp nanocrystals in the PCL matrix.

Raucci *et al*. [18] followed the sol-gel approach for the preparation of hybrid composite of HAp/PCL (25/75 w/w) by adding the PCL solution in chloroform to the Hap suspension in ethanol by mixing di-phosphorus pentoxide and calcium nitrate tetrahydrate dissolved in ethanol. The composite exhibited enhanced dispersion and good interaction between nanoHAp crystals and polymer matrix as evidenced by SEM, XRD and FTIR analysis. While the scaffolds having macropores in the size range of 200-300 µm produced by salt leaching technique fulfill the morphological requirement for osteoconduction the chemical requirement for osteoinduction is achieved by 3D surface modification of scaffolds through bio mimetic approach by growing hydroxyapatite in pre-fabricated porous scaffold using simulated body fluid. The nucleation of the mineral phase is highly

enhanced by the presence of nanoHAp particles in the PCL matrix.

The aim of the present study is to understand the effect of Tetra Hydro Furan (THF) as the medium of preparation on the phase formation and other physical parameters like particle size surface area and morphology of hydroxyapatite. This is much relevant in the synthesis of HAp-PCL composites as PCL is not soluble in aqueous media and THF is one of the acceptable solvent in processing PCL. Stoichiometric hydroxyapatite powder has been prepared in THF by precipitation method and the powder characteristics were compared with that obtained from same precursors in aqueous medium. Further nanoHAp-PCL composites in 80:20 wt%. is prepared by two routes namely solution mixing method and in situ precipitation method. The obtained composite powders were characterized for yield of product, phase and functional group identification, specific surface area and morphology of the powder obtained.

2. Material and methods

2.1. Preparation of HAp by precipitation method

Stoichiometric hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HAp) was prepared by precipitation method using deionized water (DIW) and Tetra Hydro Furan (THF) [HPCL grade; Fisher Scientific] as solvents separately. Calculated amounts of calcium nitrate tetrahydrate [Merck Mumbai] and phosphoric acid [Excelar] were used as precursors for Ca and P respectively. Ammonium Hydroxide [Merck Mumbai] was used in order to maintain the pH in the alkaline range (above 10). The flow chart of the synthesis procedure is given if Figure 1. The hydroxyapatite powders prepared in deionised water and THF are designated as HApW and HApT. These powders were further calcined at 600ºC for 1h and designated as HApWC and HApTC respectively.

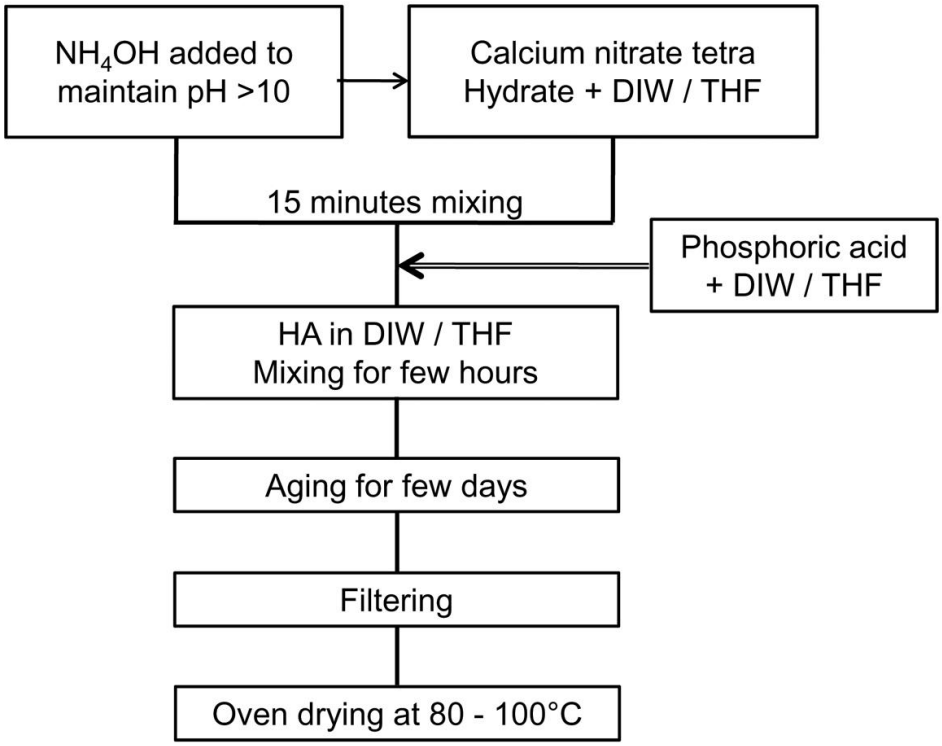


Figure 1. Flow chart for the synthesis of HAp by precipitation in DIW and THF

2.2. Preparation of nanoHAp-PCL composite by solution mixing method

Stoichiometric HAp was prepared by precipitation method using THF as solvent by following procedure as in 2.1. 2g of Polycaprolactone [M_W = 14K, Aldrich Chemicals] was dissolved in 100mL of THF and this solution was mixed with the suspension containing HAp formed by precipitation to give HAp to PCL weight ratio of 80:20. The flow chart showing the schematic representation of the preparation procedure is presented as Figure 2.

2.3. Preparation of nanoHAp-PCL composite by in-situ precipitation method

Hydroxyapatite was prepared by precipitation method by using THF as a solvent in the presence of PCL dissolved in THF to result in nanoHAp–PCL (80:20 by wt.) composite. 2g of polycaprolactone was dissolved in 100mL of THF along with required amounts of calcium nitrate tetrahydrate and ammonium hydroxide. The nanocrystals of HAp are precipitated by adding phosphoric acid solution in THF to the solution containing PCL and calcium nitrate tetrahydrate dissolved in THF. The flow chart of the preparation procedure is presented in Figure 3.

2.4. Characterization of HAp and nanoHAp-PCL composite powders

All the synthesized powders including the calcined ones were analyzed for the crystallographic phase present using the X-ray diffraction technique (M/s Bruker) using CuKα radiation. The functional group analysis was carried out using FTIR spectra. The specific surface area of the powders has been measured using surface area analyzer (Autosorb-1C, M/s Quantachrome Ltd) which follow the model of Brunauer, Emmett and Teller (BET method). The particle size distribution of the as prepared powders including the composites has been measured using Malvern Mastersizer 2000 that works on laser diffraction. The morphology of the synthesized powders was studied using Scanning Electron Microscope (Leo 440I).

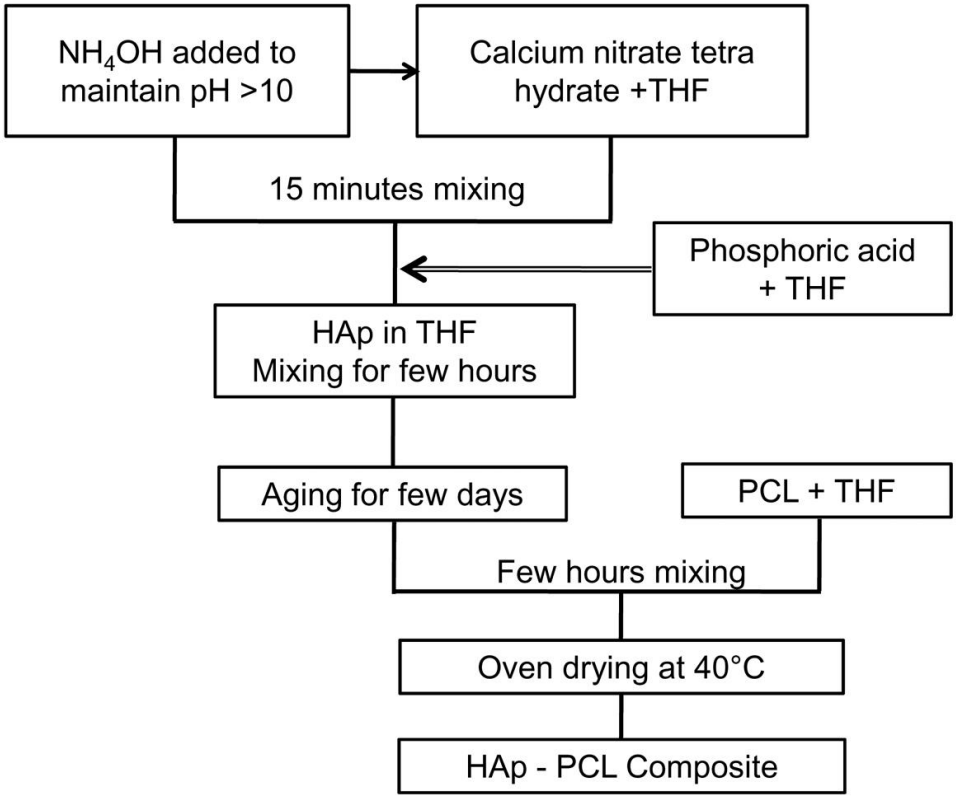


Figure 2. Flow chart for the synthesis of nanoHAp-PCL composite by solution mixing

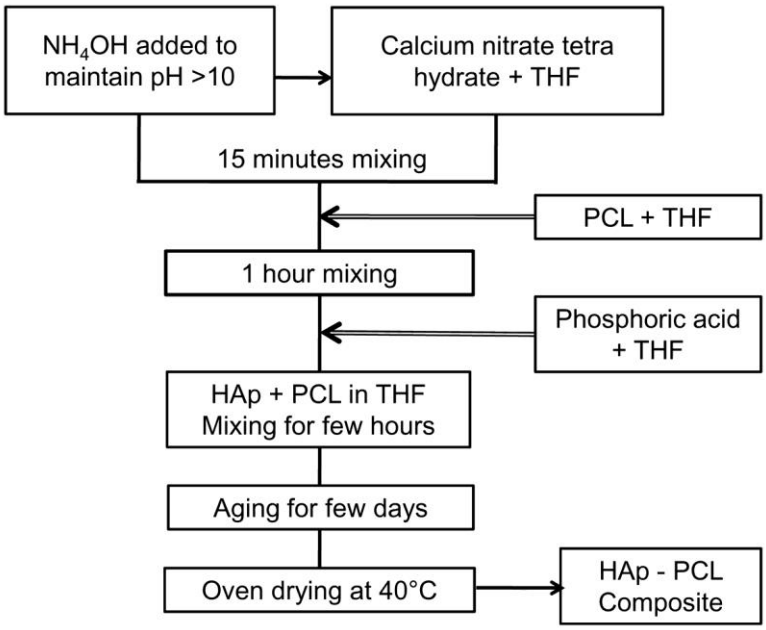


Figure 3. Flow chart for the synthesis of nanoHAp-PCL composite by in-situ precipitation

3. Results and Discussion

The preparation conditions for the synthesis of pure HAp and nanoHAp-PCL composite powders are summarized in table 1 along with the sample code designated for each powder.

Table 1: Preparation conditions of nanoHAp and HAp – PCL composite powders

Sl. No.	Material	Chemicals used	Method	Sample Code
1	HAp	CN, PA, AH, DIW	Precipitation in DIW	HApW
2	HAp	CN, PA, AH, THF	Precipitation in THF	HApT
3	HAp-PCL	CN, PA, AH, PCL, THF	Precipitation in THF and Solution mixing with PCL	HAPCL-SM
4	HAp-PCL	CN, PA, AH, PCL, THF	In situ Precipitation in PCL solution in THF	HAPCL-ISP

CN = $Ca(NO_3)_2.6H_2O$; PA = H_3PO_4; AH = NH_4OH; DIW = Deionised Water; THF = TetrahydroFuran;
PCL = Polycaprolactone, SM = Solution mixing, ISP = *In situ* precipitation

3.1. Hydroxyapatite by precipitation

Hydroxyapatite powders synthesized by precipitation in DIW and THF medium (HApW and HApT) are calcined to 600°C for 1h and designated as HApWC and HApTC. The X-ray diffraction spectra of the as prepared and calcined powders of HAp synthesized in water are presented in Figure 4(a-b) while for those synthesized in THF and calcined at 600°C for 1h are presented in Figure 5(a-b) respectively.

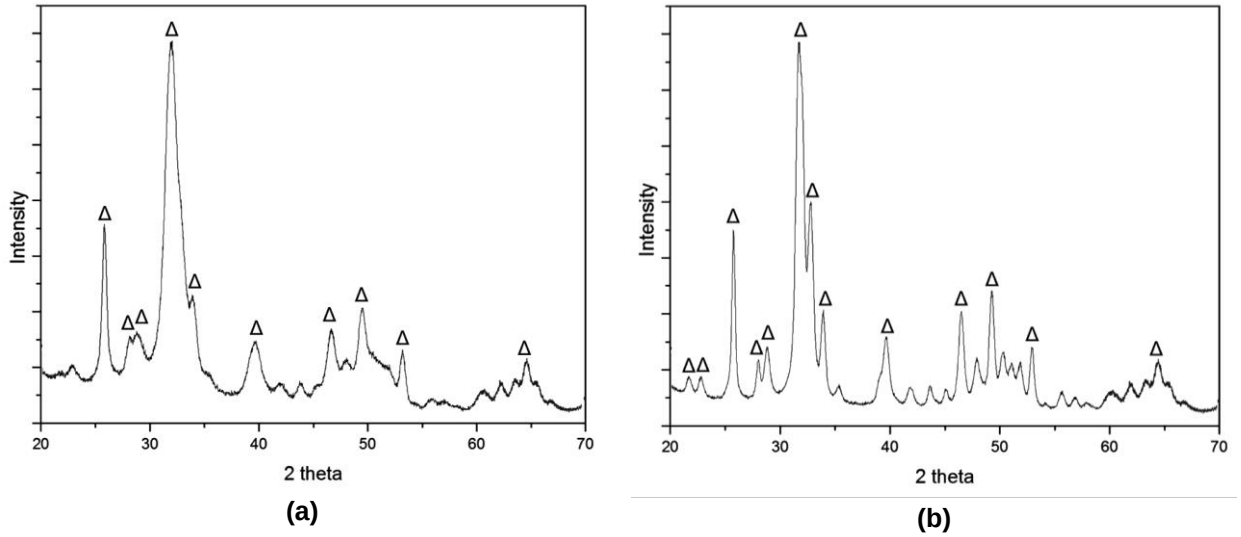


Figure 4. XRD spectra of (a) HApW and (b) HApWC; Δ = HA

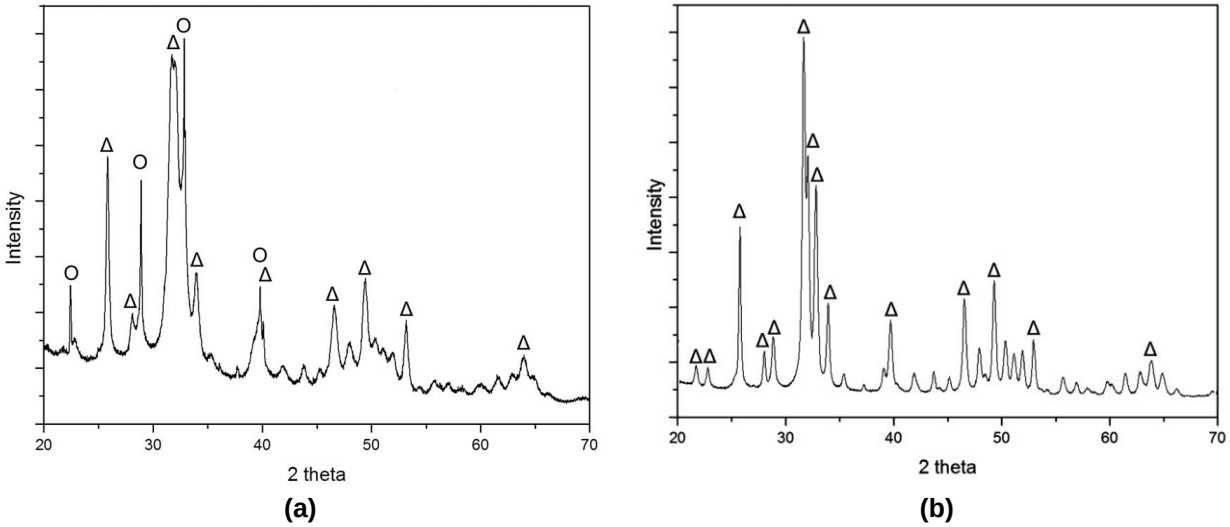


Figure 5. XRD spectra of (a) HApT and (b) HApTC; Δ = HA, ○ = Ammonium Nitrate (AN)

From the XRD spectra in Figure4(a) it can be found that the HAp powder prepared by precipitation in DIW is predominantly pure hydroxyapatite phase on comparing with the standard JCPDS card no. 9-432 and contains a small percentage of intermediate phase. The particles are in the nanocrystalline regime as the XRD pattern has broader peaks. On calcination to 600°C the hydroxyapatite phase is found stable and the crystallinity of the powder has improved as it shows slightly less broaden peak in Figure4(b) in comparison with the as prepared powder. This suggest that the high temperature leads to crystallite growth and increase in the crystallite size of the particles.

The XRD pattern for HApTin Figure5(a) is similar to that obtained for HApW in Figure4(a) proving the formation of nanocrystallites of hydroxyapatite by precipitation in tetra hydro furan medium. However, the XRD spectra show few prominent peaks other than HAp which are corresponding to ammonium nitrate(AN) precipitated due to its insolubility in THF. These peaks are generally not found during precipitation in aqueous medium as ammonium nitrate is soluble and completely removed during filtering. The ammonium nitrate is removed by washing with DIW and centrifugation successively for three times. The XRD spectra of the water washed HApT (HApTHW) presented in Figure6

show peaks only corresponding to HAp (JCPDS 9-432). The XRD spectra of HApTC powder calcined at 600°C in Figure 5(b) shows all the peaks corresponding to HAp with improved crystallinity.

The crystallite size of the as prepared and calcined powders was calculated by using Scherrer formula; $t = 0.9\lambda / B\cos\theta$, where λ is the wavelength of X-ray, B is the full width at half maximum of the highest intensity peak and θ = diffraction angle of high intensity peak. The surface area S of the powders measured by BET method and the particle size D calculated from specific surface area S using the formula; $D = 6/ (\rho \times S)$; where, ρ = density of compound are presented in Table 2 along with the crystallite size calculated by Scherrer formula. The crystallite size of the as prepared HApW and HApT (after water wash) falls in the range of 10 to 14nm and those of calcined powders falls in the range of 16 to 20nm. The HAp powders synthesized both in aqueous and non-aqueous media show high surface area and corresponding particle sizes are in the nano range. These results are in agreement with that obtained by XRD analysis. As expected the surface area values are decreased for calcined powders due to the increased crystallization and grain growth. The surface area measurements could not be conducted for HAPCL composite powders due to continuous degassing.

Table 2. Surface area, crystallite size and particle size of the Hydroxyapatite powders

Powder	Crystallite size (nm)	Surface area (m^2/g)	Particle size from SA (nm)
HApW	8	85	25
HApWC	15	50	38
HApT	10	70	28
HApTC	22	25	80

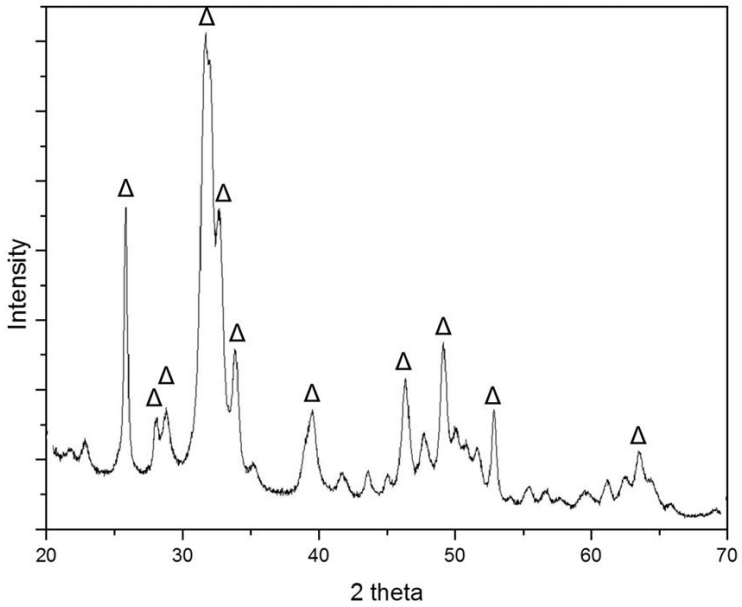


Figure 6. XRD pattern of HApTHW

The HAp powders synthesized in DIW and THF and the same powders calcined at 600°C are subjected to FTIR spectra for functional group identification. The FTIR spectra of HApT and HApTC are presented in Figure7(a-b). From the FTIR spectral analysis it is found that the hydroxyapatite powders prepared in DIW and THF medium show similar IR profiles with PO_4 bands near 566-568, 604

and 1034-1040cm^{-1} (Figure 7(a)). A shoulder at around 630 cm^{-1} observed for these samples correspond to the OH^- group. A very small intensity peak at 825 cm^{-1} seen in both the samples could be assigned to HPO_4^-. A sharp high intensity peak at 1386-1389 cm^{-1} corresponding to CO_3^{2-} group shows the presence of carbonate substituted HAp. The carbonate ions came into the system from the air during the precipitation because the reaction system was open to air. At an early stage of the phosphoric acid addition, the suspension of calcium nitrate in DI water/THF and ammonia was highly alkaline in nature. Due to this, the suspension readily adsorbed CO_2 from the air. Furthermore, the longer aging time might have prolonged the contact of CO_2 with the sample formed. All these samples show broad absorption band in the range of 3100cm^{-1} to 3600 cm^{-1} which may be attributed to the absorbed water molecules. The specific absorption band corresponding to OH^- around 3570cm^{-1} could not be identified probably as it submerged with the broad absorption peaks due to water molecules.

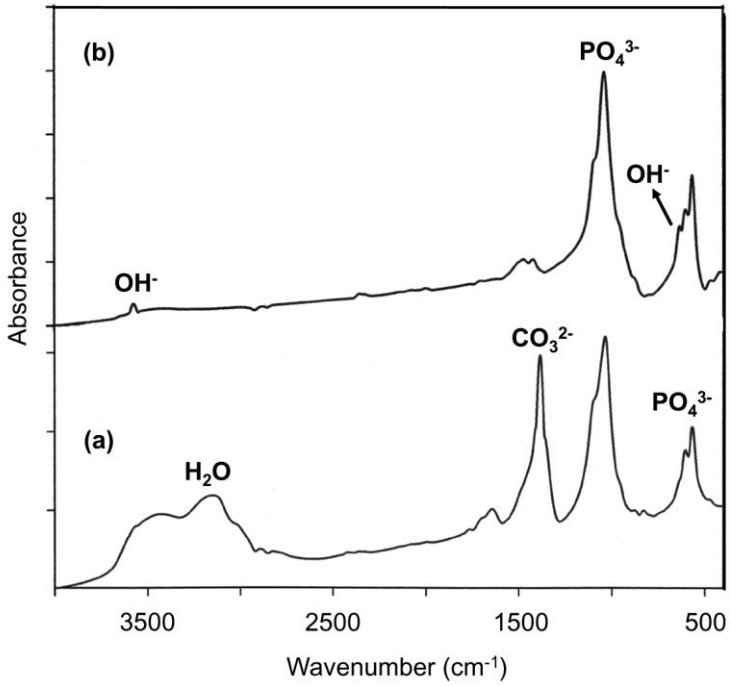


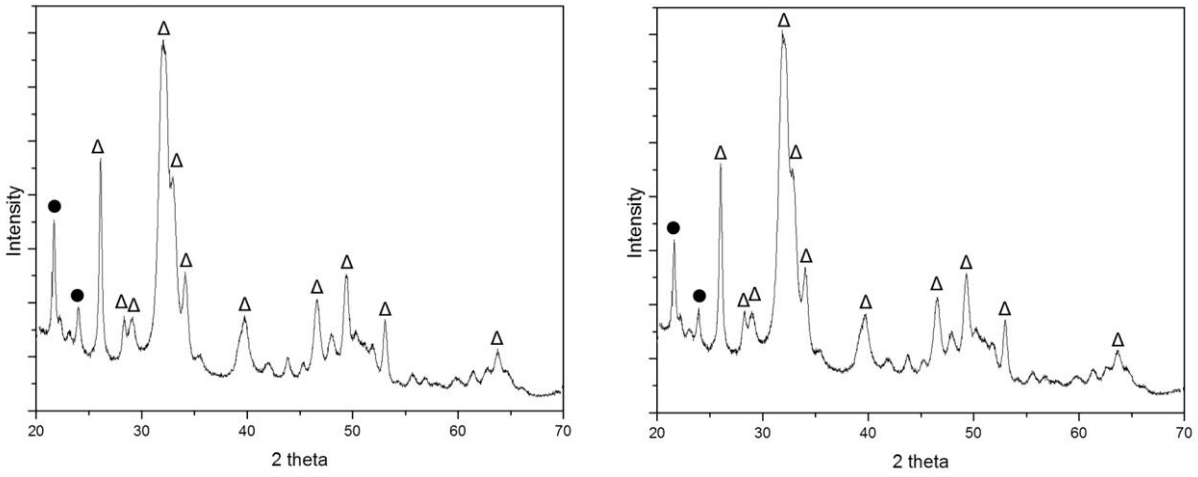
Figure7. IR spectra of (a) HApT and (b) HApTC powders

From the FTIR spectra corresponding to calcined samples HApWC and HApTC [Figure 7(b)] it can be found that the absorption peaks corresponding to PO_4^{3-} clearly exists while the intensity of peak corresponding to carbonate diminishes (1430 and 1460 cm^{-1}) due to the heat treatment they were subjected to during calcinations. The peak at 825 cm^{-1} assigned to HPO_4^- got eliminated due to conversion of calcium deficient HAp to stoichiometric HAp. Further in these calcined samples sharp peaks at around 3572-3574 cm^{-1} and shoulders around 630 cm^{-1} corresponding to vibration and stretching frequencies of OH^- ions in HAp are seen along with reduction in adsorbed water content.

3.2. Preparation of nanoHAp- PCL composite

The composite powder mixture of HAp and PCL in the weight ratio of 80:20 were prepared by two process methods namely solution mixing method (designated as HAPCL-SM) and in situ precipitation method (designated as HAPCL-ISP) as explained under experimental procedures and depicted in Figure 2 and 3 respectively. Both these composite powders are subjected to X-ray diffraction and found ammonium nitrate peaks along with diffraction peaks corresponding to HAp and PCL. The powders are washed with water for three times and centrifuged to

remove ammonium nitrate. The XRD spectra of these two powders subjected to water wash presented in Figure8(a-b) shows all the peaks corresponding to HAp (JCPDS 9-432) and two sharp diffraction peaks at 2θ = 21 and 21.9° corresponding to crystalline PCL. The broad diffraction peaks indicate nanocrystalline nature of the HAp particles precipitated which has crystallite size in the range of 10-16nm as calculated from Scherrer formula.



(a) (b)

Figure8. XRD spectra of (a) HAPCL-SM and (b) HAPCL-ISP after water wash

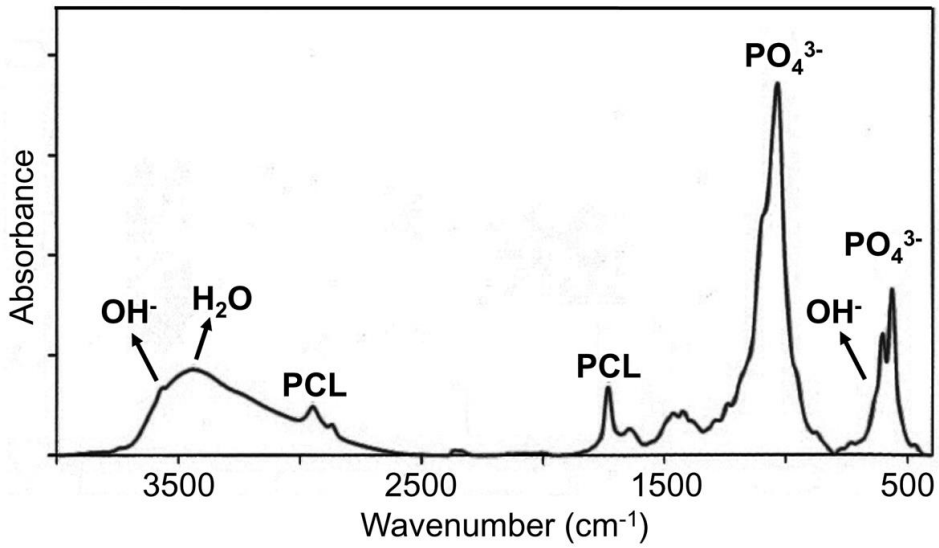


Figure 9. IR spectra of HAPCL-ISP after water wash

The HAp-PCL composite powders (HAPCL-SM and HAPCL-ISP) are subjected to IR spectroscopy and both powders show similar IR spectral graphs. The IR spectra of HAPCL-ISP powder is presented in Figure 9. The PO_4^{3-} bands were observed at 567, 602 and a strong peak at 1035-1040cm^{-1}. All these bands were sharp. A shoulder around 630cm^{-1} observed in this sample corresponds to the OH^- group. No peak corresponding to HPO_4^{2-} was observed. A very tiny carbonate peak was observed at 1414cm^{-1}. Sharp peaks at 1732 and 2947cm^{-1} are related to the major absorption peaks corresponding to polycaprolactone. A broad band at 3400cm^{-1} and a sharp peak at 3569cm^{-1} correspond to absorbed water molecules.

3.3. Particle size, surface area and morphology of HAp and nanoHAp-PCL composite powders

The particle size analysis of the synthesized powders was carried out using Master Sizer equipment. The results are presented for HApW, HApT, HAPCL-SM and HAPCL-ISP powders in Figure 10(a-d) respectively. All the samples show wider particle size distribution and which falls in the range of 1-100 microns. In HApW, 50% of the particles have size in the range of 1- 13µm, while the remaining 50% have particles in the size range of 14-100µm. Finer particles are observed in HApT, where 50% of the particles are between 1 – 8µm. The average diameter of particles inHApW and HAPT are 21µm and 19µm respectively.

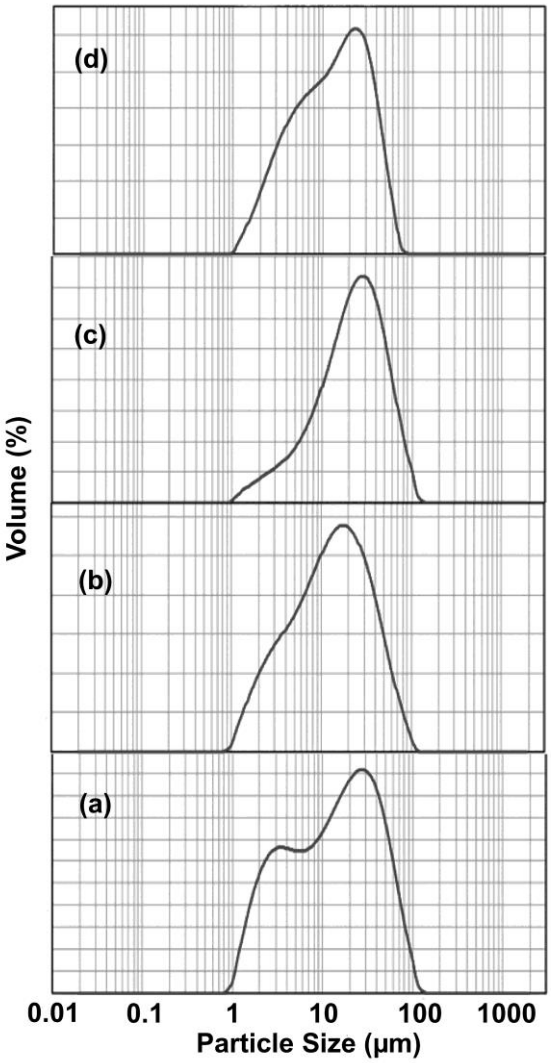


Figure 10. Particle size distribution curve for (a) HApW (b) HApT (c) HAPCL-SM and (d) HAPCL-ISP

In HAPCL-SM, 50% of particles have size in the range of 1- 20µm, while the remaining 50% have size in the range of 21-100µm. Finer particles are observed in HAPCL-ISP, where 50% of the particles are between 1 – 9µm. The rest of the particles lie between 10 – 91µm. The average diameter of particles in HAPCL-SM and HAPCL-ISP are 27µm and 17µm respectively.

The morphology of the powders HApT, HAPCL-SM and HAPCL-ISP powders could be seen in SEM of these powders presented in Figure 11(a-c).

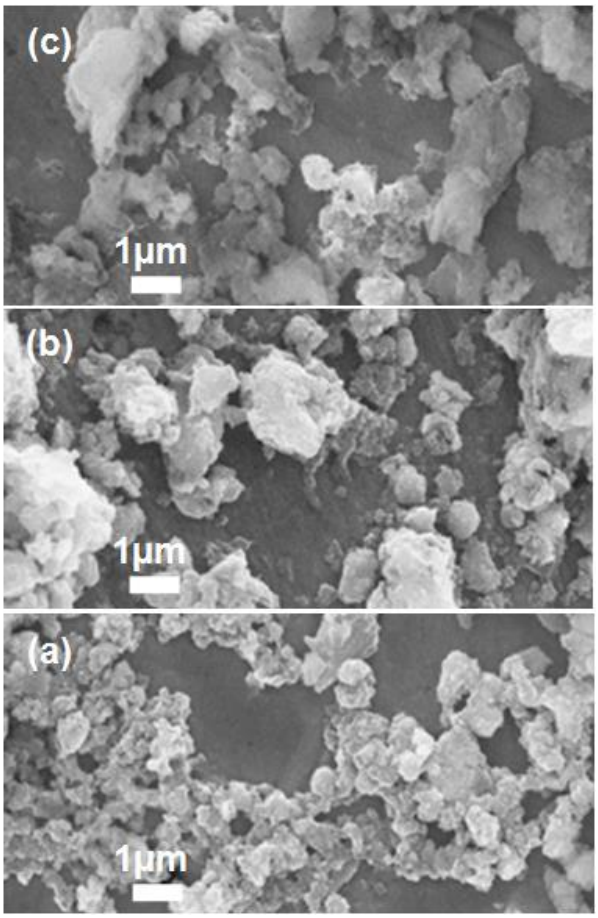


Figure 11. SEM of the powders (a) HApT (b) HAPCL-SM and (c) HAPCL-ISP

The particles are highly agglomerated in all the cases with particle size ranging from 0.5 microns to 5 microns in size. This is in good agreement with the results of particle size analysis wherein wide size distribution was observed. However, the primary particles have sizes approximately in the range of around 80 to 100 nm. Even though the particle size is expected to be in the nano range, according to the XRD results, the ultrasonic energy used to disperse the particles during preparation of the sample for SEM analysis is not enough to break down the agglomerates. The morphology of all three samples looks similar while HAPCL-SM and HAPCL-ISP in Figure 11 (b-c) bear more resemblance with one another.

In Figure 11(a) the particles of HApT seem more distinct but agglomerated whereas the particles of HAPCL composites in Figure 11(b-c) show that the polymer acts as a binding agent for the fine particles of HAp which are embedded in PCL polymer resulting in large agglomeration. Comparing the results, it may be concluded that the sizes of the agglomerates are smaller in pure HApT than in HAp-PCL composite powders, which is in agreement with the particle size analysis results.

4. Conclusions

NanoHAp having crystallite size of 10 to 20nm, primary particle size of 25 to 80nm and high surface area of 50 to 80m^2/g were successfully synthesized by precipitation method both in deionised water and tetrahydrofuran medium. The XRD and IR analysis of these powders are complementary to one another and prove the formation of HAp. HAp - PCL composites with HAp to PCL ratio of 80:20 by wt % were synthesized by solution mixing and in-situ precipitation method. These composite powders show highly agglomerated particles wherein the

nanoHAp particles are coated with PCL or are embedded in PCL matrix. The composite powders with homogeneous mixing of two biocompatible materials are expected to be potential candidate for fabricating porous scaffolds for drug delivery and tissue engineering applications.

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