RETRACTED ARTICLE: Synthesis and characterization of magnetite/hydroxyapatite nanoparticles for biomedical applications

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\textbf{ABSTRACT}

During the last few decade, magnetite/hydroxyapatite nanoparticles have attracted much attention for its potential applications in the field of medicine and controlled drug delivery. In the present work, we have intended and synthesized magnetite/hydroxyapatite nanoparticles by wet-chemical precipitation route. The thermal decomposition of the prepared sample was carried out by Thermogravimetric and Differential Scanning Calorimetry (TG/DSC) analysis upto 1000 °C; it showed that the sample was stable up to 919.2 °C. The phase formation, functional groups, morphology and magnetic property of as-prepared and annealed samples were characterized using X-ray diffraction (XRD), Fourier Transform Infrared (FTIR), micro Raman, Field Emission Scanning Electron Microscopy (FE-SEM), Transmission electron microscopy (TEM) and vibrating-sample magnetometer (VSM). By adopting Scherer method detailed structural analysis like the crystallite size, strain, dislocation density, lattice parameter, unit cell volume, surface area and fraction of crystallinity were calculated for as-prepared and annealed samples. The average crystallite size of the as-prepared, annealed at 700 °C, 800 °C and 900 °C are 39, 46, 51 and 65 nm, respectively. The dislocation density, strain and surface area were decreased. FT-IR and Raman spectra showed both as-prepared and annealed samples possessed the characteristic bands of HA\textsubscript{p}. FE-SEM and TEM images depicted and confirmed the morphological characteristics of HA\textsubscript{p}. The VSM analysis confirmed that both as-prepared and annealed samples of HA\textsubscript{p} are in diamagnetic nature. The dielectric constant of as-prepared and annealed samples decreased with increase in annealing temperature whereas the conductivity increases with increase in frequency. The antibacterial property of apatite samples depending on the types of bacterial strains and also activity changed with the crystallite size.

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1. Introduction

Medicine and surgery are the most important areas in scientific communities because of their unequivocal suggestion to human health and valued society. Every year, millions of people are suffering by bone defects caused by trauma, tumor or bone-related diseases and several were found to be dying due to the insufficiency of ideal bone tissue substitute [1]. To resolve the problem of bone defects, therapies for these defects or bone substitutes, including autografts and allograft have been developed. However autografts may be associated with donor shortage and donor site morbidity whereas allografts may have the risk of disease transmission and immune response [2]. The drawbacks of these materials are due to the potential persistence of antigenic elements in the materials; subsequently substitute synthetic materials were developed with the common objective of matching the biological and mechanical properties of human bone. In these cases, the development of biomaterials plays a very important role to promote bio implantation [3,4].

Hydroxyapatite [Ca_{10}(PO_4)_6(OH)_2 or HA_P] is a typical bioactive material. Due to its excellent osteoconductivity, biocompatibility, bioactivity, chemical, the biological similarity with the mineral constituents of human bones, teeth, it is used as bone cement, drug delivery, toothpaste additive and dental implant [5–9]. In natural human bone crystal size of HA_P is in nano-range. In recent decades, great interest has been adopted to synthesis nanosized HA_P with a grain size less than 100 nm because of its high aspect ratio and enhanced bioresorption. In addition, due to the greater surface area, nanocrystalline HA_P exhibits improved sinterability at lower temperatures resulting in a high-density body cum enhanced mechanical properties and it is suitable for biomedical applications. These primary characteristics are widely documented to majorly affect mechanical properties, biocompatibility and bioactivity. Several researchers synthesized and showed enhanced mechanical properties of Hydroxyapatite by controlling the parameters like particle size, shape, its distribution and agglomeration [10]. The constituent elements of HA_P are primarily calcium and phosphorus with a stoichiometric Ca/P ratio of 1.67 and the association of hydroxide ions are eliminated at elevated temperatures. HA_P and other related calcium phosphate minerals have been utilized extensively as implant materials for many years due to its excellent biocompatibility and bone bonding ability [11–13]. The porous structure of bio material allows the flow of extracellular fluid through the inner structure of biomedical device so as to have adequate osteoconductivity, mechanical interlocking and firm fixation of material and enhances the adhesion between the natural and synthetic bone by the formation of apatite layer [14,15].

Few literature survey has been made on the structural, optical and magnetic, behavior of hydroxyapatite nanoparticles (HAp). The characteristics of HAP depend on the methods of preparation, numerous processes for the preparation of HAP had been developed over the past one decade, including wet-chemical precipitation route solid-state reaction and hydrothermal methods etc. [16–24]. F. Mohandes and M. Salavati-Niasari [25] have interrogated that, hydroxyapatite nanostructures synthesized by simple precipitation route and studied their structural and morphological analysis. From their results the morphology of hydroxyapatite (HAP) nanostructures controlled easily with the aid of different Schiff bases. Orooji et al. [26] has investigated that Silver Iodide/Graphitic Carbon Nitride Nanocomposites by hydrothermal method. They found that Silver Iodide/Graphitic Carbon Nitride nanocomposites have great prospective for being a great antibacterial agent.

Fatemeh Mohandes, Masoud Salavati-Niasari [27] has investigated that hydroxyapatite nanostructures synthesized successfully via a complexing agent-assisted route. They prepared HA_P nanostructures with different morphologies and the shape and particle size of
HAp nanostructures were controlled by adding hydroxyacetophenone as complexing agents. Sudip Mondal et al. [28] has investigated nano-biomedical applications of Magnetic hydroxyapatite nanoparticles through facile synthetic methods and studied their shape and size, stability, and biocompatibility and magnetic saturation of nanoparticles. They introduced that, hydroxyapatite nanoparticles are used the selective treatments through guided drug delivery systems. In addition to drug delivery, the use of multifunctional MHAp in simultaneous imaging, hyperthermia, and gene/plasmid delivery is going to drastically improve the diagnosis and therapy of diseases. G. Bharat et al. [29] has investigated that MHAp/RGO nanocomposite was prepared through a hydrothermal method and studied their structural and morphological analysis. They found that, MHAp/RGO nanocomposites exhibited excellent Hb adsorption capacity and pH controlled release of the protein, indicating its potential as drug nanocarrier for biomedical application. Wei Cui et al. [30] has investigated that, the magnetite/hydroxyapatite/chitosan nanocomposites were prepared via in situ compositing method by preparing precursor solutions and molds with chitosan membrane and studied their structural, morphological and magnetic behaviour. From the magnetic measurement, it could be concluded that the nanocomposites were superparamagnetic, which is also the peculiarity of nanomagnetites. From the literature survey, it is clear that hydroxyapatite HAp nanostructure was synthesized successfully in different methods. Hence, the present work investigates the structural, optical and magnetic properties and antibacterial activity of different annealing temperature of HAp nanostructures are synthesized by wet-chemical precipitation route.

Among these processes, wet chemical co-precipitation is the most promising method because of its simplicity of experimental operations, low operating temperature and high yields of pure products [31]. During reactions, the reaction media involve no foreign elements except water. For these reasons, it is of our great interest and importance to developing the inexpensive HAp synthesis methods focused on the precise control of particle size, morphology and chemical composition [32–34]. However, it needs highly qualified and controlled parameters such as pH, temperature of solutions, working nature, composition of the starting materials, reagent concentration, addition rate, stirring technique, with speed, maturation and presence of impurities obtain the HAp monophase [35]. In the present work, a simple precipitation process using CaCl₂·2H₂O and Na₂HPO₄ (AR grade reagents Merck, India) were taken as the calcium, phosphorus precursors respectively and are dissolved separately in de-ionized water. All chemicals were directly used without further treatment. Calcium and phosphorus precursors are dissolved separately in de-ionized water.

The nanohydroxyapatite powder was prepared by wet-chemical precipitation method using calcium chloride dihydrate CaCl₂·2H₂O and di-sodium hydrogen phosphate Na₂HPO₄ (AR grade reagents Merck, India) were taken as the calcium, phosphorus

2. Materials and methods

2.1. Synthesis of pure HAp

Calcium chloride dihydrate CaCl₂·2H₂O (99% Purity) (AR grade reagents Merck, India), di-sodium hydrogen phosphate Na₂HPO₄ (98.0% Purity) NaOH (97% Purity) calcium, phosphorus precursors respectively and are dissolved separately in de-ionized water. All chemicals were directly used without further treatment. Calcium and phosphorus precursors are dissolved separately in de-ionized water.

The nanohydroxyapatite powder was prepared by wet-chemical precipitation method using calcium chloride dihydrate CaCl₂·2H₂O and di-sodium hydrogen phosphate Na₂HPO₄ (AR grade reagents Merck, India) were taken as the calcium, phosphorus
precursors respectively and are dissolved separately in de-ionized water is shown in Figure 1. The pH of both solutions was maintained 11 by adding 1 M NaOH drop by drop. The CaCl$_2$·2H$_2$O solution was added in dropwise to the Na$_2$HPO$_4$ solution while vigorously stirring and maintaining the temperature at 60 °C in 2 h. The solution was further stirred vigorously for 1 h. It was then washed three times with hot water and filtered. After filtration, the prepared samples were dried at 100 °C for 12 h and finally the nano HA$_P$ was annealed at 700 °C, 800 °C and 900 °C for 2 h to analyse the characteristics and its properties of the sample.

2.2. Characterization techniques

Differential Scanning Calorimetric (DSC) and Thermogravimetry (TG) of as-prepared HA$_P$ was carried out using a Thermal Analyzer (SDT Q600 V20.9 Build 20) over a temperature range of 30–1000 °C. X-ray diffraction (XRD) pattern data were obtained at room temperature for as-prepared and annealed samples using the powder X-ray diffraction technique (with Cu-K$_\alpha$ radiation, $\lambda = 1.5406$ Å) at 40 kV and 30 mA. FTIR spectra in the region between 4000–400 cm$^{-1}$ were obtained with a Nicolet Magna-IR 550 FTIR
The morphology of the as-prepared and annealed HAp was examined by Field Emission scanning electron microscopy (FESEM) analysis was carried out using Zeiss EVO 18-EDX special edition machine compatible with EDX machine. Transmission electron microscopy (TEM) experiments were carried out using a Technai, G2 20 Twin FEI instrument (Czech Republic). The Raman spectrum of the sample was recorded at room temperature using a micro Raman spectrometer at a source wavelength of 514.5 nm (Princeton instrument Acton sp 2500). The dielectric and alternating current conductivity properties were investigated using LCRZ-model TH2816A. Impedance properties were analyzed by using Princetone, applied research impedance analyzer of model versa STATMC frequency range from 1 Hz to 1 MHz. Vibrating sample magnetometry was carried out for as-prepared and sample annealed at 900°C. An optical absorption study was carried out by JASCO UVvis, NIR (V-670) spectrophotometer operating at a wavelength range of 200 to 2500 nm at intervals of 1 nm for all the samples.

3. Results and discussion

3.1. TG/DSC analysis

Differential scanning calorimetric (DSC) coupled with Thermo Gravimetric (TG) plot of the as-prepared powder is shown in Figure 2. The DSC is employed to determine the temperature at which the as-prepared amorphous HA\textsubscript{p} transformed into crystalline HA\textsubscript{p}. In the DSC curve of the HA\textsubscript{p}, the first endothermic peak appeared at 75.8°C was due to the evaporation of the absorbed water (residual moisture evaporation).

The endothermic bud at 424°C is ascertained due to the elimination of crystalline water in un-reacted CaCl\textsubscript{2} 2H\textsubscript{2}O and removal of Cl\textsuperscript{2–} groups. The third endothermic peak at 534.8°C corresponds to the decomposition of HPO\textsubscript{4}\textsuperscript{2–} which might be present in the chemically synthesized powder and also indicating the crystallization of HA\textsubscript{p}. The fourth endothermic peak at 665°C is due to the removal of interstitial water from HA\textsubscript{p} [36]. The fifth endothermic peak at 792°C indicated the decomposition of carbonate.
into CO\(_2\) [37]. In the TG, three discrete weight-loss regions occurred as is seen in Figure 2. The first region at 85.9\(^\circ\)C with 14.1\% weight loss corresponds to the elimination of physically adsorbed water. The second weight loss regions appear in between 85.9–781.1\(^\circ\)C in the TG plot with a 2.55\% weight loss which corresponds to the elimination of lattice water. The third weight loss observed in the TG diagram at 781.1–919.2\(^\circ\)C with a weight loss of 3.03\% and an endothermic peak at 795\(^\circ\)C in DSC curve correspond to the removal of interstitial water and the decomposition of carbonate into CO\(_2\) (\(CaCO_3 \rightarrow CaO + CO_2\)). A broad and shallow endothermic shoulder above at 919\(^\circ\)C is due to decomposition of HA\(_p\) take place. This observation confirmed by M.D. Adak and K.M. Purohit [38].

3.2. X-ray diffraction (XRD) analysis

Figure 3 shows the XRD patterns of the HA\(_p\) annealed at different temperatures such as as-prepared, 700, 800 and 900\(^\circ\)C. The peaks of all four diffraction patterns were compared with standard HA\(_p\) of the international center for diffraction data (ICDD card # 09-432). The intensity and d-spacing of XRD patterns are good agreement with the standard hexagonal HA\(_p\) structure. The calculated lattice parameters and unit cell volume of as-prepared HA\(_p\) is almost equal to the standard values are given in Table 1 within brackets. Particularly the strong intensive diffraction planes at (211), (112) and (300) confirmed that all the as-prepared and annealed samples had a hexagonal structure similar to that of pure HA\(_p\) [39]. This is confirmed that, the different annealing temperatures not influenced in the composition of the HA\(_p\) structure of the as-prepared sample. It is cleared from XRD pattern, the as-prepared HA\(_p\) had a poor crystalline phase together with the amorphous phase. Also, the overlapped doublet plane at (211), (112) indicated the presence of carbonated in HA\(_p\) [40]. The line broadening of (211), (112) and (300) reflections were used to calculate the crystallite size, dislocation density, strain, surface area and the fraction of crystallinity with the aid of simple equations and are given in Table 2. As the
annealing temperatures increased XRD peaks became more distinct and width of the peaks became narrower. The distinction of XRD peaks indicated that the increasing fraction of crystallinity. The decreased width of XRD peaks indicated the increase in the crystallite size. It is observed that the crystallite size increased from 39.23 to 65.39 nm when annealed from as-prepared to different elevated temperature. Dislocation density, strain and surface area were decreased with increased in annealing temperature. The slight shifts of the diffraction lines toward the lower angle (2θ), when the temperature is raised might be due to increase in lattice parameters. From the obtained XRD pattern planes of the lattice parameters were calculated by the following formula [1].

\[ d^2 = \frac{4}{3} \left( \frac{h^2 + h k + k^2}{a^2} \right) + \frac{I^2}{c^2} \]  
(1)

where d is the interplanar spacing, a and c are lattice parameters and h k l are miller’s indices. For the determination of the unit cell volume, the following formula is used [20].

\[ V = \frac{\sqrt{3}}{2} a^2 c \]  
(2)

The crystallite size D was determined using the well-known Scherrer relation [21].

\[ D = \frac{0.9\lambda}{\beta \cos \theta} \]  
(3)

where \( \lambda \) is the wavelength of radiation, (is the full width at half-maximum (FWHM) and \( \theta \) is the diffraction angle [22]. The average particle size is 39, 46, 51 and 65 nm for as-prepared, annealed at 700°C, 800°C and 900°C, respectively. Calculation shows that the particle size increases with increasing annealed temperature.

The microstrain \( \varepsilon \) was determined by using the following formula [22]

\[ \varepsilon = \frac{\beta \sin \theta}{4} \]  
(4)

It is observed that micro-strain decreased from \( 0.962 \times 10^{-3} \) to \( 0.550 \times 10^{-3} \) as the increase of annealing temperature. In general, variation in micro-strain may be due to the change in microstructure, size and shape of the particles.

| Sample | Parameter a (Å) | Parameter c (Å) | Unit cell volume (Å³) |
|--------|----------------|----------------|----------------------|
| a      | 9.475          | 6.845          | 532.189              |
|        | (9.418)*       | (6.884)*       | (528.80)*            |
| b      | 9.509          | 6.848          | 536.288              |
| c      | 9.528          | 6.865          | 538.233              |
| d      | 9.541          | 6.872          | 541.816              |

| Sample | Crystallite size (nm) | Dislocation density \( \times 10^{15} \) (lines/m²) | Strain \( \times 10^{-3} \) (line -2 m -4) | Surface area \( \times 10^{11} \) m² line -1 | Fraction of crystallinity (Xc) |
|--------|-----------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|---------------------------------|
| A      | 39.23                 | 0.824                                        | 0.962                                        | 5.261                                        | 1.921                           |
| B      | 46.70                 | 0.483                                        | 0.756                                        | 4.135                                        | 2.642                           |
| C      | 51.36                 | 0.401                                        | 0.687                                        | 3.750                                        | 3.470                           |
| D      | 65.39                 | 0.259                                        | 0.550                                        | 3.006                                        | 7.702                           |
The dislocation density \( \rho \) can be determined from the following relation \([23,41]\).

\[
\rho = \frac{1}{D^2}
\]  

(5)

The fraction of crystallinity \( X_c \) of the HAp nanoparticles was calculated from the equation

\[
X_c = \left( \frac{0.24}{\beta} \right)^3
\]  

(6)

Specific surface area \( S \) of the HAp nanoparticles was determined by the formula \([42]\)

\[
S = \frac{6 \times 10^3}{D\rho}
\]  

(7)

where \( \rho \) is the theoretical density of \( \text{HA}_p \) (3.16 g/cm\(^3\)).

### 3.3. FT-IR analysis

FT-IR spectra of as-prepared and annealed at different temperatures are shown in Figure 4 and whose corresponding tentative frequencies are shown in Table 3. The FT-IR spectra of as-prepared \( \text{HA}_p \) showed a large number of bands corresponding to different vibrational modes of different functional groups. Most of these bands were attributed to the different modes of functional groups present in pure \( \text{HA}_p \). A few additional bands might be due to some impurities present in \( \text{HA}_p \). Weak bands observed at 1582, 1417 and 875 cm\(^{-1}\) were due to \( \text{CO}_3^{2-} \), which were due to absorption of atmospheric carbon dioxide during the preparation of \( \text{HA}_p \) \([43,44]\). Bands at 3438 cm\(^{-1}\) and 1640 cm\(^{-1}\) were due to O-H stretching and H-O-H bending modes of lattice water molecule \([45]\). The band at 1640 is also observed in all annealed samples with a slight shift is due to an availability of
OH–H present in the HAp. Symmetric stretching and the librational modes of OH– appeared at 3571 cm\(^{-1}\) and 624 cm\(^{-1}\), respectively in the FT-IR spectrum of HAp [39]. As the annealing temperature increased, the OH– group of the transmittance peak decreased due to the removal of OH– group [46]. The band at 624 cm\(^{-1}\) is absent in the FT-IR spectra of samples heated at higher temperatures. Bands appeared at \(\sim660, 472, 1041–1088\) and 563–605 cm\(^{-1}\) are the characteristic bands for \(v_1, v_2, v_3\) and \(v_4\) stretching modes of PO\(_4^{3-}\) ions, respectively. The band at 3571 cm\(^{-1}\) as well as 624 is assigned due to the stretching mode or vibration of hydroxyl groups in the crystal structure of HAp which are not observed at elevated temperature [36,47]. From this analysis, one can confirm the formation of HAp. Carbonate ions can substitute for either OH– (or) PO\(_4^{3-}\) ions in the apatite structure [36]. This result all of these phosphate bands were present in spectra indicating the presence of the HAp structure on our samples. Since carbonates are constituents of bone structures, the presence of CO\(_3^{2-}\) may improve the bioactivity of HAp rather than being a cause of concern. These results are once again confirmed by earlier reports of HAp nanoparticles [48].

### 3.4. FE-SEM analysis

The Surface morphologies FE-SEM images of as-prepared and annealed at 700, 800 and 900 °C HAp are shown in Figure 5(a)–(e). FE-SEM images give the direct information about the shape and size of the grains present in the samples. After the heat treatment, the mean particle size and morphology changes can be clearly noticed from FE-SEM micrographs. The as-prepared sample showed fine grains with homogeneous, uniform distribution and highly porous network structure. It also showed the agglomeration of particles in the as-prepared sample. In the annealed samples at 700, 800 and 900 °C were having a cylindrical and hexagonal morphology and non-uniform grain sizes. The grains size ranging from 103–344, 172–526 and 275–789 nm for the samples annealed at 700, 800 and 900 °C, respectively. The crystal growth of the powder can be visually seen when the powder was annealed at 900 °C. It is evident from the FE-SEM and the XRD results as the annealing temperature increased the grain size in FE-SEM and the crystallite size
calculated from XRD are increased. In thermally treated HA_p particles are well separated and they are uniformly distributed. As the particles are well separated the number of microspores is increased when compared to as-prepared HA_p. In the sample annealed at 900 °C, the grains of rod-like hexagonal shape crystals were presented. The EDX spectrum of the synthesized powder was shown in Figure 5(e) revealed the elemental composition of nano-HA_p. Clear peaks corresponding to Ca, P and O were obtained, no other significant elements detected. Through this analysis, the Ca/P ratio was calculated to be approximately 1.70, confirming the purity of HAP.

Figure 5. FE-SEM micrograph images of HAp at different annealing temperatures (a) as-prepared, (b) 700 °C, (c) 800 °C, (d) 900 °C and (e) EDX spectrum of as-prepared HA_p.
3.5. TEM analysis

TEM images of hydroxyapatite nanoparticles annealed at 900 °C were presented in Figure 6(a) and 6(b). The images confirmed the formation of nanocrystalline powder. The TEM image clearly revealed that the morphology of HA is fine nanometric spherical and equiaxial particles. The particles are uniform and their size ranged from 193 to 700 nm due to agglomeration. The morphology of the particles is observed to be almost non-uniform (irregular) hexagonal structure. Fine discrete particles of nano range are clearly visible in the loosely agglomerated powder. It is further revealed that the individual particles are composed of small crystallites. The size of these crystallites lies between 179–384 nm. The nano crystals exhibited sharp edges and corners. The rate of nucleus formation and the rate of growth are related to the concentration of reactants, upper saturation, temperature, stirring rate, etc. [49]. The selected area electron diffraction (SAED) pattern is shown insert Figure 6(b). The spots and ring patterns indicated the polycrystalline nature of hydroxyapatite, however, it also showed that random orientation due to the agglomeration of platelets. This TEM observation of the shape of particles is good agreement with the earlier FE-SEM analysis.

3.6. Micro Raman spectra analysis

The micro Raman spectra of as-prepared and different temperature annealed HAp samples are shown in Figure 7(a)–(d). The assignments of Raman scattering bands are summarised and presented in Table 4. As-prepared HA showed amorphous nature with less crystalline. When the annealing temperature increases, the crystallization also increases in all the samples. The main Raman scattering characteristic peak at 962 cm\(^{-1}\) is due to PO\(_4^{3-}\) (P-O) presented in all the samples [50]. Notwithstanding the characteristic peak is very weak in the as-prepared sample. When the annealing temperature increased, the PO\(_4^{3-}\) peak became stronger, but CO\(_3^{2-}\) band at 1119 cm\(^{-1}\) became weaker. The weak and broadband at \(v_3\) 1054 cm\(^{-1}\), \(v_4\) 583 cm\(^{-1}\) and \(v_2\) 429 cm\(^{-1}\) modes of the PO\(_4^{3-}\) are also active evidence the characteristic of HAp.
Carbonate bands in the Raman spectra are very weak due to its low concentration. The most intense band at 962 cm$^{-1}$ is assigned to the $v_1$ mode of the $\text{PO}_4^{3-}$ group for crystalline HA$_p$. The $\text{PO}_4^{3-}$ bands are observed at 429, 459 cm$^{-1}$ is ascertained bending modes, $v_2$—O–P–O, $v_4$—O–P–O of vibrations at 583 cm$^{-1}$. A small medium bud at n583 cm$^{-1}$ is due to out plane bending mode of $v_4$—O–P–O Raman modes of vibrations. Which is complemental analysis of FT-IR. A sharp Raman scattering shift at 1054 cm$^{-1}$ is ascertained owing to triply degenerative asymmetric stretching modes of $v_3$—PO$_4^{3-}$ (P–O bond) and $v_1$—symmetric stretching mode of CO$_2^{2-}$.

| As-prepared | 700$^\circ$C | 800$^\circ$C | 900$^\circ$C | Peak assignment | References |
|-------------|--------------|--------------|--------------|----------------|------------|
| $-$         | 429          | 459          | 583          | $v_2$ doubly degenerate bending mode of $\text{PO}_4^{3-}$ (O–P–O bond) | [38,44]    |
| $-$         | 429          | 459          | 583          | $v_4$ triply degenerate bending mode of $\text{PO}_4^{3-}$ (O–P–O bond) | [39]       |
| $-$         | 429          | 459          | 583          | $v_4$ triply degenerate bending mode of $\text{PO}_4^{3-}$ (O–P–O bond) | [44]       |
| 962         | 962          | 962          | 962          | $v_1$ non-degenerate symmetric stretching mode of $\text{PO}_4^{3-}$ (P–O bond) | [38,41,44] |
| 1054        | –            | 1035         | 1035         | $v_3$ triply degenerate asymmetric stretching mode of $\text{PO}_4^{3-}$ (P–O bond) and $v_1$—symmetric stretching mode of CO$_2^{2-}$ | [38,41,44] |
| 1119        | 1119         | 1119         | 1119         | $v_1$ symmetric stretching mode of CO$_2^{2-}$ | [41]       |

Figure 7. Micro Raman spectra of HA$_p$ at different annealing temperatures (a) as-prepared, (b) 700$^\circ$C, (c) 800$^\circ$C and (d) 900$^\circ$C.

Table 4. Micro Raman frequency assignments of as prepared, different temperature annealed HA$_p$.
lower Raman scattering shift approximately 19 cm\(^{-1}\). Which is due to either asymmetric stretching vibration of \(v_3-\text{PO}_4^{3-}\) or symmetric stretching modes of \(\text{CO}_3^{2-}\). Which is granting the Ca/P ratio of prepared HA\(_P\). Which is on-par result of FT-IR. Furthermore, the band located at 1119 cm\(^{-1}\) is the characteristic signature of \(\text{CO}_3^{2-}\) substitution in HA\(_P\) and is assigned to the \(v_1\) mode of the \(\text{CO}_3^{2-}\) group [51,52].

3.7. Optical study

Figure 8 shows the Optical absorption study of as-prepared and annealed at 700, 800 and 900°C HA\(_P\) have carried out in the wavelength range of 200 to 2500 nm at intervals of 1 nm. The optical energy band gap (E\(_g\)) can be calculated by using an equation [53]:

\[
\alpha = \frac{c}{h\nu} (h\nu - E_g)^n
\]

where E\(_g\) is the optical energy band gap and \(h\nu\) is the photon energy obtained by drawing a line between \((\alpha h\nu)^{1/n}\) and photon energy \(h\nu\). The index number \(n\) is taken as \(1/2\) for the allowed direct band to band transitions in sample. The calculated optical band gaps of as-prepared and annealed at 700, 800 and 900°C HA\(_P\) are 3.86, 3.84, 3.54, and 3.03 eV, respectively. The redshift of the optical band gap could be obtained by decreasing the value of the optical energy gap with increased in annealing temperature. The redshift is due to the grow of grain size, increased lattice parameters (XRD analysis) and the decreased in defect states near the bands which in turn the value of band gap decreased [54,55]. The optical energy band gap of as-prepared HA\(_P\) showed that its nature is dielectric. The decreased in optical band gap with increased in annealing temperature of HA\(_P\) showed that its dielectric nature decreases and also it had good agreement with the dielectric study.

**Figure 8.** Optical energy band gap of HAp at different annealing temperatures (a) as-prepared, (b) 700 °C, (c) 800 °C and (d) 900 °C.
3.8. Electrical analysis

3.8.1. Dielectric analysis

The study of the dielectric property is an elegant experimental tool to probe the electrical properties of a crystalline solid. The analysis of dielectric characteristics indicates the response of the grown material into an applied electric field. Variation of dielectric constant can be attributed to the different type of polarization. The dependence of the frequency of the ac field is studied at room temperature in the frequency range of 50 Hz to 200 kHz. For the dielectric measurement, each prepared sample was made into the discs of 13 mm diameter and 1 mm average thickness separately. The capacitance and the dielectric loss of all the samples are obtained experimentally and the dielectric constant ($\varepsilon'$) is calculated by using the relation as given below [56].

$$\varepsilon' = \frac{Ct}{\varepsilon_o A}$$

where $C$ is the capacitance of the sample, $\varepsilon_o$ is the permittivity of the vacuum, $A, t$ are the area and average thickness of the disc, respectively.

Figure 9 represents the variation of dielectric constant with frequency at room temperature. The decrease in value of dielectric constant with increase in frequency is attributed to the electrical relaxation processes or due to the inability of the dipole to comply with alternating field and hence the dipoles lag behind the frequency of the applied field. This decrease becomes less towards high and very high frequencies. The frequency dependence can be divided roughly in the region above 0.75 kHz in which, the dielectric constant only has a small decrease towards higher frequencies. Below 0.75 kHz exhibit a steep increase of dielectric constant towards low frequency. The change in dielectric constant affects the spreading of electromagnetic fields in bone fractures. Thus the spreading of electromagnetic fields may make a contribution to the healing of bone fractures so it is very important to control dielectric constant of HA [56].

**Figure 9.** Dielectric constant as a function frequency of HAp at different annealing temperatures (a) as-prepared, (b) 700 °C, (c) 800 °C and (d) 900 °C.
The Variation in the dielectric loss with frequency is shown in Figure 10. For the as-prepared sample initially, the loss increases with frequency up to 100 kHz and then it is almost constant. All the annealed samples showed a rapid increase in the dielectric loss with frequency and then decreased gradually. The high dielectric loss at low frequencies is attributed to oscillations of the dipole. At higher frequency, ionic polarization ceases, hence energy is not spent to rotate the dipole of the ions present in HA\(\text{P}\) so the dielectric loss is found to be lower [57]. As the annealing temperature increased dielectric constant decreased. This indicates that there is an inverse proportion between dielectric constant and crystallite size [58].

### 3.8.2. AC conductivity

The plot of frequency vs. ac conductivity for as-prepared and annealed at 700, 800 and 900 °C HA\(\text{P}\) samples at room temperature is shown in Figure 11. The ac conductivity is calculated from the dielectric data using the relation [59].

\[
\sigma_{ac} = 2\pi f\varepsilon'\varepsilon_0 \tan \delta
\]

It is noticed that the ac conductivity increased with increase in frequency for all samples. But for as-prepared HA\(\text{P}\) the increase in ac conductivity is maximum than that of annealed samples. As the annealing temperature increased the conductivity decreased for a given applied frequency. The as-prepared sample may contain a higher number of free dipoles so huge number of electrical polarization takes place, hence it shows higher conductivity than that of annealed samples. The calculated ac conductivity values are in the order of \(10^{-5}\) S m\(^{-1}\) so dielectric nature of hydroxyapatite is confirmed as referred by Omer Kaygili et al. [60].
The graphical representation of frequency vs. impedance for as-prepared and annealed at 700, 800 and 900 °C samples at room temperature is shown in Figure 12. The impedance (Z) value of all the samples decreased steeply up to 200 kHz and then decreased slowly with the increase in frequency up to 1 MHz. The sample which is annealed at 900 °C showed maximum impedance at low frequencies than the as-prepared and other annealed samples. It directly reflects the conductivity result that is as the annealing temperature increased conductivity decreased but impedance increased.

3.9. Vibrational sampling magnetometer

The magnetic behavior of the as-prepared and annealed HAp at 900 °C are measured using a vibrating sample magnetometer (VSM) at room temperature with respect to the applied field from −3 to +3 T at room temperature are shown in Figure 13. The magnetization curve clearly indicated that magnetization increased linearly with the external field in the opposite direction. The magnetic moment was developed with the external field in the opposite direction indicate the diamagnetic nature of the as-prepared and annealed HAp sample [61]. The slope, which represents the magnetic susceptibility (χ) was negative and constant for both the as-prepared and annealed HAp also confirm the diamagnetic nature of the sample [62].

3.10. Antibacterial activity

The antibacterial activity of the as-prepared and annealed at 700, 800 and 900 °C hydroxyapatite powder’s, are carried out by the well diffusion method against the bacterial
strains Gram-negative *K. Pneumonia, S. dysenteriae, E. coli, P. aeruginosa, and P. Vulgaris* as well as Gram-positive *S. aureus and S. pneumonia* on Mueller Hinton agar, according to the Clinical and Laboratory Standards Institute (CLSI) [63]. The media plate’s Mueller Hinton agar (MHA) was streaked with bacteria 2–3 times by rotating the plate at 60° angles for each streak to ensure the homogeneous distribution of the inoculums. After
inoculation, discs (6 mm Hi-Media) loaded 1.5 mg/ml and 1.75 mg/ml, of the test samples, were placed on the bacteria-seeded well plates using micropipettes. The plates were then incubated at 37°C for 24 h. The inhibition zone around the well was measured and recorded. Amoxicillin (Hi-Media) was used as the positive controls against (K. pneumonia, S.aureus, S.dysenteriae, E. coli, P. aeruginosa, S. pneumonia and P. vulgaris) bacteria, respectively. The positive controls results were as compared to that of the efficacy of the test as-prepared and annealed HA nanoparticles at 700, 800 and 900°C.

The resultant data were given in Tables 5 and 6. These data revealed that the zone of inhibitions changed with the bacterial strains and the antibiotic. Samples containing antibiotic (erythromycin) showed higher activity than the pure HA nanoparticles. In the antibiotic mixed samples, S. Pneumonia and S.dysenteriae bacteria showed highest antibacterial activity for as-prepared sample annealed at 800°C, respectively. S. Pneumonia showed highest antibacterial activity for both erythromycin loaded and pure as-prepared HA nanoparticles. It was obvious that the antibacterial property on apatite samples was dependent on the types of bacterial strains, annealing temperature and loaded antibiotic. Therefore, we recommend using these HA nanoparticles as an antibacterial agent for S. Pneumonia and can potentially be applied in medical and environmental fields. It was reported that the Gram positive bacteria had a relatively thick wall composed of many layers of peptidoglycan polymer, and only one membrane (plasma membrane). The Gram negative bacteria had only a thin layer of peptidoglycan and a more complex cell wall with two cell membranes, an outer membrane, and a plasma membrane. The addition of the outer membrane of the Gram negative bacteria cells influenced the permeability of many molecules. Under certain conditions, the Gram-negative bacteria were more resistant to many chemical agents than Gram-positive cells [64]. The results in Tables 5 and 6 also showed that the gram negative bacteria were slightly more resistant than gram positive bacteria to the action of HA nanoparticles. The bactericidal effects of nano HA nanoparticles are due to the production of highly reactive oxygen species (OH−, H2O2 and O2−) on the surface of the HA nanoparticles linked with fatal damage to the bacteria. In fact, the formation of H2O2 is the

### Table 5. Zone of inhibition as-prepared and annealed at 700, 800 and 900°C without antibiotic.

| Bacteria       | As-prepared | 700°C | 800°C | 900°C | Amoxicillin |
|----------------|-------------|-------|-------|-------|-------------|
| K. pneumonia   | 11          | 10    | 15    | 12    | 11          |
| S. aureus      | 13          | 11    | 11    | 09    | 12          |
| S. dysenteriae | 12          | 10    | 11    | 11    | 13          |
| E. coli        | 14          | 15    | 16    | 14    | 11          |
| P. aeruginosa  | 12          | 10    | 11    | 11    | 12          |
| S. pneumonia   | 15          | 11    | 16    | 17    | 11          |
| P. vulgaris    | 14          | 14    | 13    | 13    | 13          |

### Table 6. Zone of inhibition as-prepared and annealed at 700, 800 and 900°C with an antibiotic.

| Bacteria       | As-prepared | 700°C | 800°C | 900°C | Amoxicillin |
|----------------|-------------|-------|-------|-------|-------------|
| K. pneumonia   | 17          | 16    | 17    | 16    | 16          |
| S. aureus      | 15          | 16    | 15    | 14    | 11          |
| S. dysenteriae | 16          | 15    | 20    | 14    | 11          |
| E. coli        | 15          | 16    | 16    | 13    | 12          |
| P. aeruginosa  | 16          | 14    | 17    | 14    | 09          |
| S. pneumonia   | 20          | 14    | 15    | 15    | 09          |
| P. vulgaris    | 15          | 14    | 16    | 13    | 10          |
primary effect that contributes to the antibacterial activity, which takes place through penetration of $\text{H}_2\text{O}_2$ via the cell walls. Another possible explanation for the antibacterial effect, according to Devanand Venkatasubbu et al., it was based on the abrasive surface ordering (i.e. texturing) of HA$_\text{P}$ due to surface defects and aggregates which contribute to the mechanical damage to the cell membrane of the bacteria [65].

4. Conclusion

Nanocrystalline HA$_\text{P}$ was successfully synthesized by wet-chemical precipitation route. Annealing plays a really significant part in moderating the various physical as well as chemical properties of HA$_\text{P}$. The thermal properties of the synthesized HA$_\text{P}$ are assigned by TG/DSC analysis show the thermal stability of the material. XRD pattern revealed that the crystal structure of as prepared and annealed HA$_\text{P}$ is similar to that of the hexagonal HA$_\text{P}$ and also shows the phase purity and crystallinity of HA$_\text{P}$. XRD results of the samples indicate that Crystallite size, Dislocation density, Strain, Surface area, fraction of crystallinity, cell parameters and unit cell volume were affected by the annealing temperature. The FTIR analysis confirms functional groups and it indicates the carbonate ion content is largely reduced with the increase in annealing temperature. The micro Raman analysis supports the FTIR spectra. EDAX spectra confirm the presence of elements in HA$_\text{P}$. The TEM images confirm the morphologies of HA$_\text{P}$ obtained by FE-SEM. VSM results show that both as-prepared and annealed sample present the diamagnetic nature. As annealing temperature increases the decrease in optical band gap shows the increase in electrical conductivity and decrease in impedance. The UV result has a very good agreement with the electrical study results. Antibacterial activity results show that hydroxyapatite is biocompatible when the antibiotic is added with HA$_\text{P}$ its biocompatibility increases. The biocompatibility of HA$_\text{P}$ changes with annealing temperature.

Disclosure statement

No potential conflict of interest was reported by the authors.

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