Effect of stirring rate on the size of hydroxyapatite nanoparticles synthesized by a modified heat-treated precipitation method

Eduardo Hernández-Silva¹, Fabiola Vázquez-Hernández², Salvador Mendoza-Acevedo³, Mario Pérez-González⁴, Sergio Tomás-Velázquez⁵, Patricia Rodríguez-Fragoso⁵, Julio Mendoza-Álvarez⁵, Juan Pedro Luna-Arias⁶,*

¹Nanoscience and Nanotechnology Ph.D. Program, Center for Research and Advanced Studies of the National Polytechnic Institute, Alcaldía Gustavo A. Madero, C.P. 07360, Mexico City, Mexico
²Mexico City Autonomous University, C.P. 07160, Mexico City, Mexico; Army and Air Force University, Military School of Engineers, Lomas de San Isidro, Miguel Hidalgo, C.P. 11200, Naucalpan, State of Mexico, Mexico
³Center of Nanoscience, Micro, and Nanotechnology of the National Polytechnic Institute, Alcaldía Gustavo A. Madero, C.P. 07738, Mexico City, Mexico
⁴Mathematics and Physics Academic Area, Institute of Basic Sciences and Engineering, Autonomous University of Hidalgo State, C.P. 42184, Mineral de la Reforma, State of Hidalgo, Mexico
⁵Department of Physics, Center for Research and Advanced Studies of the National Polytechnic Institute, Alcaldía Gustavo A. Madero, C.P. 07360, Mexico City, Mexico
⁶Department of Cell Biology, Center for Research and Advanced Studies of the National Polytechnic Institute, Alcaldía Gustavo A. Madero, C.P. 07306 Mexico City, Mexico

Received 15 October 2022; Received in revised form 7 February 2023; Accepted 21 April 2023

Abstract

Chemical synthesis is one of the most employed methods to obtain crystalline hydroxyapatite nanoparticles. It is feasible to vary the synthesis conditions and study their effects on nanoparticle structure. In this work, hydroxyapatite nanoparticles were synthesised by a modified heat-treated precipitation method and varying stirring rate of the solution during the nucleation process. The main goal was to reduce the particle size without affecting the crystallinity degree of the synthesized material which is important for several use cases, such as biomedical applications. The produced materials were characterized by X-ray diffraction, Fourier transformed infrared spectroscopy, µ-Raman spectroscopy, X-ray photoelectron spectroscopy, high-resolution transmission electron microscopy and scanning electron microscopy. The nanoparticle’s size decreased by increasing the stirring rate during the nucleation step, showing that the employed synthesis method is efficient for obtaining hydroxyapatite nanoparticles of variable sizes without affecting the crystallinity degree.

Keywords: hydroxyapatite nanoparticles, modified precipitation synthesis, structural characterization

I. Introduction

Hydroxyapatite is a calcium phosphate compound [(Ca)₉(P₂O₄)₆(OH)₂] having a hexagonal crystal structure with space group P6₃/m and lattice parameters \( a = b = 9.42 \text{ Å} \) and \( c = 6.88 \text{ Å} \) [1]. Good properties, such as bioactivity, biocompatibility, solubility, sinterability, adsorption, osteoconductivity, anti-toxicity and anti-immunogenicity, enable its diverse applications such as catalysis, formulation of pharmaceutical products, separation techniques by chromatography, water treatment and biomedical applications, including dental engineering, bone tissue regeneration and drug delivery systems [2]. In biomedical investigations, hydroxyapatite nanoparticles (nHAp) have been used as transport vehicles for genes, proteins and drugs; therefore, to
achieve the best possible efficiency as transport vehicles toward cancer and other cells it is essential to synthesize porous nHAp with specific morphologies on nanoscale level [3].

Different methods have been developed for synthesizing hydroxyapatite powders with different morphologies, including aerosol pyrolysis, combustion, coprecipitation, microemulsion, hydrothermal reaction, microwave-assisted hydrothermal reaction, sol-gel, solid-state reaction and wet chemical precipitation [4–12]. Low-temperature synthesis processes result in structures with a high degree of crystallinity and reduced size, avoiding biosorption or other degradation processes, which makes the material viable for diverse biomedical applications [4]. These synthesis methods offer the possibility of manipulating the physicochemical properties of HAp or the design of compounds that provide a particular biological interaction, presenting a variety of formulations for various applications, having advantages over multiple types of drugs that have been used in several in vivo studies [5,6]. The synthesis, properties and applications of materials with sizes less than 100 nm and the high surface/volume ratio of nanoparticles are the key characteristics that make them useful in biomedicine due to the improvement of properties, functionalization capacity and conjugation of biomolecules, among other features [5,7]. Desirable characteristics of nHAp can be obtained by sol-gel synthesis method since it is easy to control different processing parameters. Thus, Salimi et al. [13] varied the agitation rate and temperature to determine their influence on the particle size. However, hydrothermal and wet chemical precipitation methods are the easiest and most effective methods to obtain crystalline nHAp. The chemical precipitation method is also used to synthesize nHAp with a hexagonal structure, using a solution that emulates biofluids [14].

Different variables used for the synthesis of hydroxyapatite through the chemical precipitation method have been tested to determine their influence on the nanomaterial’s crystallinity, morphology and dispersibility. The studied variables included synthesis temperature, pH, drying methods and the use of ethanolamine as an additive, and showed a small effect on the shape of the nanoparticles, but a significant effect on their dispersibility [15]. The influence of other additives, such as cetyltrimethylammonium bromide (CTAB), on nucleation process during the hydrothermal method has also been studied [16]. This surfactant is used for regulating the nucleation process and, therefore, the crystal growth through interacting with precursor phosphate ions through charges and stereochemical complementarity. Thus, the phosphate molecules can be incorporated into the existing nuclei at a steady rate to control the nanoparticles’ size and shape at a certain temperature [16]. Polyamidoamine (PAMAM) dendrimers were also tested during the hydroxyapatite synthesis using the hydrothermal technique, acting as a nucleation site by binding calcium ions through the amine or amide groups located on their surface. Thus, rod- or ellipsoid-like nanoparticles can be produced by controlling the coordination capacity of the dendrimers as a function of their generation number [17]. Furthermore, characteristics of nHAp prepared using hydrothermal method were also evaluated by varying the dripping rate of the calcium source [18].

In this work, we assessed the stirring rate during the nucleation process in a modified heat-treated precipitation method, representing an experimental condition previously not evaluated for this synthesis method. The synthesis of nHAp was carried out by varying the stirring rate to determine whether this modification reduces the particle size without affecting their crystallinity for future use as a drug delivery system.

II. Experimental

The chemicals used in this work were dibasic potassium phosphate trihydrate (K$_2$HPO$_4$·3H$_2$O), cetyltrimethylammonium bromide (CTAB), potassium hydroxide (KOH), calcium chloride (CaCl$_2$) and ethanol (EtOH). All these chemical reagents of analytical grade were purchased from Sigma-Aldrich and used without further purification. Milli-Q (Millipore) water (ddH$_2$O) was used to conduct the experiments.

2.1. Synthesis of hydroxyapatite

The nHAp were synthesized by a modified heat-treated precipitation method with variation of stirring rate during the nucleation process. The aim was to determine the influence of this process parameter on the material’s characteristics. First, 2.4 mmol of K$_2$HPO$_4$·3H$_2$O and 4.5 mmol of CTAB were dissolved in 100 ml of degassed ddH$_2$O at room temperature in a Kitasato flask connected to vacuum. The solution was heated to 50°C and the pH was adjusted to 12 with 1 M KOH and kept under gentle magnetic stirring for 2 h. Next, 60 ml of a 0.33 M CaCl$_2$ solution were added dropwise at a flow rate of 20 ml/h, maintaining gentle magnetic stirring. Once the addition of salt was completed, the colloidal solution was incubated at 90°C for 12 h under magnetic stirring, maintaining constant stirring rates during the nucleation process, i.e. 0, 100 and 200 rpm. Then, the precipitate was sequentially washed three times with ddH$_2$O and 70% ethanol and dried at 90°C for 24 h in a vacuum oven. Finally, the synthesized material was homogenized in a porcelain mortar and stored at room temperature (RT).

2.2. Characterization of synthesized nHAp

The crystalline structure of all nHAp samples was determined by X-ray diffraction using a Bruker-D5000 X-ray powder diffraction system (SIEMENS) from the Physics Department at the Center for Research and Advanced Studies (Cinvestav). A Cu Ka X-ray emission line with a wavelength of 1.5418 Å was used, and 2θ
angle was scanned from 20° to 70° at a scanning speed of 0.02° to 2° min⁻¹. To determine the crystal size of the samples, the Scherrer equation was used:

\[ D = \frac{k \cdot \lambda}{\beta \cos \theta} \]  

(1)

where \( D \) is the crystallite size, \( k \) is the shape factor of the crystal, its value is generally 0.89 for ceramic materials, \( \lambda \) is the wavelength of the ray source, \( \beta \) is the half width of maximum intensity and \( \theta \) is the Bragg angle [19].

The identification of functional groups was performed by the Fourier transformed infrared spectroscopy using a Nicolet 6700 FT-IR Spectrometer (Thermo Scientific) from the Physics Department at Cinvestav, before the analyses, each sample powder was compacted in a KBr pellet.

µ-Raman spectroscopy was performed in a LabRAM HR800 Confocal Micro Raman Spectrometer (Horiba Jobin Yvon) in the Center of Nanosciences and Micro and Nanotechnologies from the National Polytechnic Institute (CNMN-IPN). An argon laser (453–514 nm) of 10 mW was used and the samples were analysed at RT without special preparation. The scanning was performed in the wavenumber range of 100 to 1800 cm⁻¹.

Elemental composition of the samples was determined by X-ray photoelectron spectroscopy using a K-Alpha XPS Spectrometer (Thermo Scientific) in the Physics Department at Cinvestav, supplied with a monochromatic Al Kα X-ray source (1486.6 eV). The beam size was set to 400 μm in diameter. Scanning electron microscopy (SEM) analysis was performed with a Quanta FEG 250 SEM System (Thermo Fisher) in CNMN-IPN. The samples were covered with carbon using an SPI-Module carbon coater and deposited on carbon sample holders, and SEM micrographs were collected with the microscope operating at a voltage of 10 kV.

Transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HRTEM) images were obtained with a JEOL JEM-ARM200F microscope CNMN-IPN. The powders were dispersed in isopropanol by sonication, deposited on carbon-coated copper meshes and dried at RT. TEM micrographs were collected with the microscope operating at a voltage of 200 kV.

2.3. Data processing

The OriginPro 8 (OriginLab), Digital Micrograph v2.31 (Gatan Inc.), Prism 6 (GraphPad) and Avantage v4.88 (Thermo Scientific) software packages were used for data processing. ImageJ 1.52a software (National Institutes of Health, NIH) was used for particle size measurements.

III. Results and discussion

The hydroxyapatite nanoparticles were synthesized using a modified heat-treated precipitation method and varying stirring rate (0, 100 and 200 rpm) of the solution during the nucleation process. The aim was to determine the influence of this parameter on the nanoparticle characteristics since numerous groups have previously tested other conditions.

X-ray diffraction analyses were performed to determine the crystal structure and crystallite sizes (\( D \)) of each sample (Table 1). Diffractograms (Fig. 1) showed the characteristic patterns of the hydroxyapatite hexagonal crystal structure according to the JCPDS crystallo-

Table 1. Synthesis conditions and characteristics of the produced hAp

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stirring rate</th>
<th>( D ) [nm]</th>
<th>Ca/P</th>
<th>Particle length [nm]</th>
<th>Particle width [nm]</th>
<th>Miller indexes</th>
<th>( d ) [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM1</td>
<td>0 rpm</td>
<td>4.2</td>
<td>1.70</td>
<td>48 (±2)</td>
<td>19 (±0.4)</td>
<td>300</td>
<td>2.728</td>
</tr>
<tr>
<td>HM2</td>
<td>100 rpm</td>
<td>3.7</td>
<td>1.68</td>
<td>28 (±0.8)</td>
<td>12 (±0.3)</td>
<td>112</td>
<td>2.759</td>
</tr>
<tr>
<td>HM3</td>
<td>200 rpm</td>
<td>3.7</td>
<td>1.66</td>
<td>26 (±1)</td>
<td>11 (±0.3)</td>
<td>300</td>
<td>2.776</td>
</tr>
</tbody>
</table>

135
E. Hernández-Silva et al. / Processing and Application of Ceramics 17 [2] (2023) 133–139

Figure 2. FT-IR spectra of the hydroxyapatite samples: a) HM1, b) HM2 and c) HM3 (the vibrational modes of the functional groups are marked with dashed lines)

Figure 3. µ-Raman spectra of the hydroxyapatite samples: a) HM1, b) HM2 and c) HM3 (the vibrational modes of the phosphate group are marked with dashed lines)

graphic chart (00-009-0432). The main peaks were located at the 2θ angles of 31.65°, 32.13° and 32.76°, corresponding to the (211), (112) and (300) planes, respectively. Other important characteristic Miller indices of crystalline hydroxyapatite were also identified and correspond to the planes (002), (210), (202), (310), (222), (312) and (213). The average crystallite size $D$ of the synthesized nHAp decreases with the increase of stirring rate, from 4.2 to 3.7 nm (Table 1), however crystallinity is not changed.

Characterization of the hydroxyapatite samples by FT-IR spectroscopy was performed to verify the presence of the characteristic vibrational modes of the hydroxyapatite functional groups (Fig. 2). A weak absorption band was detected at 473 cm$^{-1}$, which was assigned to the O–P–O symmetric bending mode. The strong bands seen at 571 and 602 cm$^{-1}$ correspond to the asymmetric O–P–O bending mode of the phosphate group $\text{PO}_4^{3-}$ and the symmetric O–H bending mode, respectively, while the weak band at 632 cm$^{-1}$ was assigned to the O–H vibrational mode. The band at 960 cm$^{-1}$ was assigned to the symmetric P–O stretching mode. Two strong bands at 1030 and 1085 cm$^{-1}$ correspond to the asymmetric P–O stretching mode of the $\text{PO}_4^{3-}$ group, while the band at 1637 cm$^{-1}$ was assigned to the O–H bending mode. Two other strong signals identified at 3412 and 3572 cm$^{-1}$ were assigned to stretching of the hydroxyl group $\text{OH}^-$ and free O–H stretching mode, respectively. Additionally, three bands corresponding to carbonate were identified; the signal at 880 cm$^{-1}$ corresponds to the asymmetric bending mode of the $\text{CO}_3^{2-}$ group, while the signals at 1420 and 1458 cm$^{-1}$ were assigned to the asymmetric C–O stretching. Carbonate formation was attributed to the adsorption of atmospheric $\text{CO}_2$ during the synthesis process, which is related to high alkaline conditions in the solution, where hydroxyl ions react with $\text{CO}_2$ to generate carbonate ions $\text{CO}_3^{2-}$. The FT-IR results confirm the formation of crystalline hydroxyapatite particles by the modified heat-treated precipitation synthesis.

To confirm the presence of the characteristic functional groups of the hydroxyapatite samples, the samples were analysed by µ-Raman spectroscopy. This technique is complementary to FT-IR and XRD, as it provides precise information on the vibrational modes of the ions and atoms that form molecules and crystal lattices, thereby obtaining chemical and structural information on the analysed material. The spectra of all the samples (Fig. 3) show the presence of bands at 430, 448, 578, 591, 608, 959, 1044 and 1075 cm$^{-1}$, all corresponding to group $\text{PO}_4^{3-}$. Unlike the FT-IR spectra, the type A band for $\text{CO}_3^{3-}$ at 1106 cm$^{-1}$ is not present and the type B band for $\text{CO}_3^{2-}$ at 1070 cm$^{-1}$, if it is present, may be hidden by the $\text{PO}_4^{3-}$ band at 1076 cm$^{-1}$ [20,21]. The bands shown in the spectra also confirm the results obtained by FT-IR analyses.

XPS spectra revealed the elemental composition of the hydroxyapatite samples, showing the presence of the elements Ca, O, P and C (Fig. 4). The stoichiometric ratios Ca/P obtained by this analysis are shown in Table 1. The data revealed that Ca/P ratio of the prepared HAp samples decreases from 1.70 to 1.66 when stirring rate
increases to 200 rpm and is close to the theoretical value of 1.67 [18,22].

The morphology and surface characteristics of the different hydroxyapatite samples were determined by SEM (Fig. 5), which indicates that agglomerates of primary particles are present in all three powders. Homogeneous structure can be recognised and it seems that higher degree of agglomeration was obtained at higher stirring rate.

TEM analyses revealed a rod-like morphology in all the hydroxyapatite samples (Fig. 6). The average dimensions were determined by measuring the length and width of 300 particles observed in several images obtained by TEM, employing the ImageJ 1.52a software; the values were $48(\pm2)\,\text{nm} \times 19(\pm0.4)\,\text{nm}$, $28(\pm0.8)\,\text{nm} \times 12(\pm0.3)\,\text{nm}$ and $26(\pm1)\,\text{nm} \times 11(\pm0.3)\,\text{nm}$ for the HM1, HM2 and HM3 sample, respectively (Figs. 6a,e,i). HR-TEM results also confirmed the crystalline nature of the nHAp (Figs. 6b,f,j), where the crystalline planes correspond to the measured interplanar distances for each sample, revealing the hexagonal crystalline phase of hydroxyapatite as shown in the XRD analyses. Images obtained for the HM1 and HM2 powders (Figs. 6c,d and 6g,h, respectively) show (112) and (300) crystalline planes and their interplanar distances, while the images obtained for HM3 (Figs. 6k,l) show (210) and (211) crystalline planes.

Our results showed a decrease in the size of hydroxyapatite nanoparticles when the stirring rate increased during the nucleation process without affecting the crystallinity. The elemental composition and Ca/P ratio of the samples are similar to the previously reported data [22,23]. The previous works have mainly focused on studying other synthesis parameters, including the impact of the pH, temperature, surfactant and drying method on the morphology, crystallinity and dispersibility of nHAp. Thus, at pH = 10, the average length of nHAp was 30 to 50 nm and at higher pH the dispersibility gradually decreases. Crystallinity degree rises significantly when the temperature is increased, whereas the lower temperature favours the formation of amorphous hydroxyapatite. Increasing the chemical precursor’s concentration generates nanoparticles with higher lengths and diameters, but slightly lower Ca/P ratios. Polyethylene glycol induces the formation of HAp nanorods with a larger aspect ratio at high-temperatures; polysorbate 20 promotes the formation of small-sized nanorods and D-sorbitol can stimulate the formation of long nanorods at low synthesis temperature. Additionally, CTAB induces the formation of rod-shaped nHAp. Drying methods have shown effects on the morphology and dispersibility of nHAp. Vacuum dried particles have homogenous morphology but low dispersibility. Particles desiccated by freeze-drying are bigger and have a high degree of crystallinity and good dispersibility compared to the nanoparticles obtained by atmospheric or vacuum drying at room temperature [15,16,24–26].

To reduce the particle size of HAp, some studies have already reported variations of the stirring temperature and stirring rate, joint with the increased sintering temperature for obtaining nHAp with good mechanical properties and excellent surface as microbial immobilizers [27]. The effect of the stirring rate in the hydrothermal process or the typical wet chemical precipitation has not been studied in previous works, but it was confirmed that particle size and crystallinity can be...
The implementation of stirring in the nucleation step contributes to the decreased size of the nHAp synthesized by the modified heat-treated precipitation synthesis without affecting their morphology, chemical composition and crystallinity, representing a novel contribution to nHAp synthesis to obtain particle sizes less than 30 nm. These characteristics are essential for the functionalization and coupling processes of various compounds, such as drugs, biomolecules, antibodies, and immunofluorescent markers, which may have biomedical applications as drug delivery systems.

Acknowledgments: The authors thank Marcela Guerrero Cruz from the Department of Physics (Cinvestav-IPN), Luis Alberto Moreno Ruiz, Raul Borja Urby, and Hugo Martinez Gutiérrez from the Center for Nano Sciences and Micro and Nano Technologies (National Polytechnic Institute), for their support in the characterization of samples. Eduardo Hernández Silva acknowledges Conacyt Mexico for his Ph.D. fellowship (483638). This work has been supported by the Instituto de Ciencia y Tecnología del Distrito Federal (ICyTDF) (grant PINV11-48), now renamed Secretaría de Educación, Ciencia, Tecnología e Innovación de la Ciudad de México (SECTEI) to Dr. Juan Pedro Luna Arias, as well as institutional funding from the Department of Cell Biology (Cinvestav-IPN) to Dr. Juan Pedro Luna Arias.

References


7. K. Chatterjee, S. Sarkar, K.J. Rao, S. Paria, “Core/shell nanoparticles in biomedical applications”, Adv. Colloid In-


