Facile methods of preparing pure hydroxyapatite nanoparticles in ordinary laboratories

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ABSTRACT
The present work features some simple methods of preparing pure hydroxyapatite nanoparticles (nano-HAp), a useful biomaterial, via various wet chemical methods and using biogenic sources such as eggshells and animal bone. The nano-HAp hence prepared was subsequently characterized by FTIR spectroscopy and XRD technique. The FTIR spectra confirmed the presence of PO₄³⁻ and OH⁻ ions as major functional groups in the prepared material, with some additional peaks implying the presence of CO₃⁻ ions and adsorbed water molecules. The XRD patterns, in agreement with the JCPDS 09-432 data, demonstrated the crystalline nature of the nano-HAp and confirmed the phase as being apatite. The average grain diameter of the nanocrystallites was found in the range of 15-30 nm. The preparatory methods depicted herein can be easily employed in ordinary high school laboratories having basic facilities such as availability of distilled water, some handy glasswares, common laboratory chemicals and instruments such as balance, hot air oven and furnace.

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1. Introduction
Hydroxyapatite (HAp), with the chemical formula Ca₁₀(PO₄)₆(OH)₂, is one of the most attractive bioceramics for several biomedical applications due to its osteoconductive and antimicrobial properties and biocompatibility as well as lack of toxic and inflammatory responses [1-4]. HAp is the major mineral constituent of human bone accounting for approximately 65 weight fraction of the latter. Bone is, in fact, a nanocomposite material comprising HAp mineral and collagen proteins, its properties thus largely being dependent on the nanoscale morphology. The apatite crystals are the essential parts of tooth and bone of all the vertebrates. When used as implants, the synthetic HAp is able to provide a scaffold or template for new bone regeneration and growth, and supports the osteoblast adhesion and proliferation [3,4].
Such an important bio-ceramic can be synthesized either from natural sources such as bone, corals, eggshells, body fluids, etc. or via various chemical routes including sol-gel method, mechano-chemical precipitation, hydrothermal technique, flame spray and microwave assisted method, and others [2-16].

The major mineral constituents of human bone are calcium and phosphorous with traces of other substances [17,18]. However, a considerable amount of carbonate (CO$_3^{2-}$) also appears to be present in the bone. The presence of CO$_3^{2-}$ ions in biological apatite is of great importance because it is the main source of lattice distortion, creating micro-stresses and crystalline defects in its surrounding area which, in turn, play a vital role in its solubility. Thus, synthetic apatite should exhibit small particle size and presence of CO$_3^{2-}$ [18].

It has been demonstrated that the nanocrystalline HAp (nano-HAp) exhibits enhanced biocompatibility, bioactivity and mechanical performance over microcrystalline or bulk HAp, which has been attributed to their unique quantum confinement effects and large surface area to volume ratio. Thus the nano-HAp can be used in designing superior biocompatible coatings for the implants and in developing high strength composite materials [4,12,17,18].

With the dawn of nanotechnological revolution during last few decades, and in context of unparalleled opportunities offered by these technological advances also in developing countries, the facile methods for nanomaterials synthesis are of tremendous scientific importance. There is overwhelming interest in the versatile routes for the synthesis of such materials among young scientists in their early career. Taking into account the vigor of scientists of new generation towards nanomaterials and their technological implications, the objective of present work has been to demonstrate simple methods of preparing nano-HAp in ordinary high school laboratories also using wet chemical methods by utilizing biogenic waste as resources. As evidence for the success of the methods used, the characterization of the materials by Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) are reported here.

2. Experimental Section

2.1 Materials

Analytical grade calcium nitrate tetrahydrate, di-ammonium hydrogen phosphate, ammonium hydroxide (25%), ortho-phosphoric acid (85%), urea, calcium oxide, ethanol and ethylene-diamine-tetraacetic acid (EDTA) were purchased from Merck India Ltd. Egg shells and adult buffalo femoral bones were collected from local market in Kathmandu. All solutions were prepared in distilled water.

2.2 Preparation of nano-HAp

Four different methods used for the preparation of nano-HAp were sol-gel method, precipitation method using calcium oxide and ortho-phosphoric acid as starting materials, precipitation method using aqueous slurry comprising of egg-shells powder and ortho-phosphoric acid solution, and calcination of animal bone.

(a) Sol-gel Method

This method was adopted from different research articles [11,12,19,20] published elsewhere. In this method, 10.8 g of calcium nitrate tetrahydrate, Ca(NO$_3$)$_2$.4H$_2$O and 3.89 g of diammmonium hydrogen phosphate, (NH$_4$)$_2$HPO$_4$ were separately dissolved in 50 mL of water taken in a 100 mL conical flask for each. Phosphate solution was then added drop-wise to the calcium nitrate solution maintained at temperature of 75 °C. The pH was maintained at 11 throughout the experiment using ammonia solution. The resulting solution was continuously stirred for 2 hrs and the product was allowed to cool and precipitate for 24 hrs. The aged gel thus obtained was filtered and washed with doubly distilled water and ethanol several times till the filtrate was neutral. Finally, the resulting clear white powder was stored in a hot air oven.
maintained at 85 °C for 12 hrs for further analyses.  

\[
10\text{Ca(NO}_3\text{)}_2 + 6(\text{NH}_4)_2\text{HPO}_4 + 8\text{NH}_3\text{OH} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 20 \text{NH}_4\text{NO}_3 + 6\text{H}_2\text{O}
\]

(b) Precipitation Method

This method was adopted on the basis of works reported elsewhere [2,5,17,21,22]. Calcium oxide (CaO), ortho-phosphoric acid (H_3PO_4), and ammonium hydroxide (NH_4OH) were used as starting substances for this method. First of all, 7.955 g of dry CaO powder was added to 50 mL of water taken in a 100 mL conical flask and vigorously stirred at 20 °C for 24 hrs to form aqueous suspension of Ca(OH)_2. Then 9.732 g of 85% H_3PO_4 was slowly added to the solution at a rate of about 1.5 mL/min. The reactants were further stirred for 24 hrs to attain the maturation stage, under constant stirring. The pH of the solution was maintained at 10 using ammonia solution. The precipitate was filtered, washed and dried in hot air oven at 85 °C for 12 hrs. The chemical reaction may be written as:

\[
3\text{CaO} + 3\text{H}_2\text{O} \rightarrow 3\text{Ca(OH)}_2
\]

\[
3\text{Ca(OH)}_2 + 3\text{H}_3\text{PO}_4 \rightarrow 3\text{CaHPO}_4 + 6\text{H}_2\text{O}
\]

\[
5\text{Ca(OH)}_2 + 3\text{H}_3\text{PO}_4 \rightarrow \frac{1}{2}\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 9\text{H}_2\text{O}
\]

The reaction can be represented as:

\[
\text{CaCO}_3 \rightarrow \text{CaO} + \text{CO}_2
\]

\[
10\text{CaO} + 10\text{H}_2\text{O} + 6\text{H}_3\text{PO}_4 \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 18\text{H}_2\text{O}
\]

(d) Animal Bone as Starting Material

This method was adopted from different research papers [16,26-28]. Briefly, the femoral bones of buffalo was collected from butcher shop that were then cut into small pieces and boiled in water for 3 hrs. The bone was then washed with acetone for several times to remove fats and other impurities and hence dried at 160 °C for 48 hrs. The pieces were ground to powder with particle diameter less than 450 µm. The powder was treated with 4M NaOH (solid-liquid weight ratio of 1.40) for deproteinization. The content was heated at 250 °C for 5 hrs.

The defatted and deproteinized powder was calcined at 700 °C for 6 hrs in a muffle furnace to obtain grayish white powder of HAp. The powder was further calcined at 1000 °C for another 6 hrs to study the effect of calcination temperature.

2.3 Methods for characterization
Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR analysis was performed by using a Prestige-21 FTIR Spectrometer (Shimadzu Company, Japan). The spectra were collected in the range of 4700-400 cm\(^{-1}\) using KBr pellet method.

X-Ray Diffraction (XRD)

The crystal phase and structure of the samples were determined by Bruker D2 Phaser X-ray diffractometer with a monochromatic CuK\(\alpha\) radiation source (\(\lambda = 0.15418\) nm) with 2θ angles ranging from 20° to 80°. The accelerating voltage of 30 kV and emission current of 10 mA were used.

3. Results and Discussion

The phase purity and presence of major functional groups of the HAp powder were attested by FTIR analyses. Fig. 1 shows the FTIR spectrum of the HAp powder chemically synthesized by sol-gel method.

The spectrum is dominated by the typical PO\(_4^{3-}\) bands of crystalline apatite phase characterized by the peaks representing triply degenerate \(\nu_3\) (PO\(_4^{3-}\)) asymmetric mode centered at 1021 cm\(^{-1}\) and 1087 cm\(^{-1}\) (shoulder), non-degenerate symmetric stretching mode \(\nu_1\) (PO\(_4^{3-}\)) at 962 cm\(^{-1}\) and components of the triplet of \(\nu_4\) (PO\(_4^{3-}\)) bending modes at 560 cm\(^{-1}\) and 470 cm\(^{-1}\) [2,6,12,21,23,29,30]. The broad band located between 3200-3600 cm\(^{-1}\), together with weak and broad band around 1627 cm\(^{-1}\) of H-O-H bending mode indicates the presence of absorbed water. The weak peak located at 3570 cm\(^{-1}\) corresponds to the vibrations of OH\(^-\) ions in the HAP lattices [6,24,26].

The two peaks centered near 1490 and 1426 cm\(^{-1}\) are assigned to the \(\nu_3\) vibration mode and the weak peak at about 870 cm\(^{-1}\) is due to the \(\nu_2\) vibration mode of free, planar CO\(_3^{2-}\) ions (group symmetry D\(_3\)H) [12,20,23-25]. The weak band at 2360 cm\(^{-1}\) can be attributed to C=O bonds from adsorbed atmospheric carbon dioxide [25].

In summary, the FTIR spectrum shown in Fig. 1 verifies, in consistence with several literatures, the success of the chemical synthesis of the HAp powder. Fig. 2 shows a closer look on structure of the raw eggshell and the HAp.

The IR spectrum of the raw eggshell shows the strong carbonate peaks centered at 1490 cm\(^{-1}\) and 870 cm\(^{-1}\), and the sharp peak located at 710 cm\(^{-1}\), which represents the absorbance by Ca-O bond [31].

The weak band at 2360 cm\(^{-1}\) is attributed to C=O bonds from carbonate [25,32] while the broad band at around 2863 cm\(^{-1}\) appears due to OH stretching vibration and that around 3600 cm\(^{-1}\) arises due to N-H bonds due to proteins present in the raw eggshells.
The IR spectrum of the HAp powder prepared from eggshells given towards the bottom of Fig. 2 is much different from the spectrum of raw eggshells as expected but contains the noteworthy peaks related to the HAp phosphates such as those centered around 470, 560, 1020, and 1090 cm\(^{-1}\), as compared with Fig. 1.

Fig. 3 shows the FTIR spectra of buffalo bone powder and the HAp prepared from it. Unlike in the eggshells, the spectrum of the buffalo bone powder, as shown in upper part of Fig. 3, clearly shows the presence of phosphate peaks centered at 1090, 1040, 603, 568 cm\(^{-1}\) [10,32,33] as well as OH group at 3570 cm\(^{-1}\) [12,26,34] confirming the presence of hydroxypatite as a major mineral component in the buffalo bone.

The presence of sharp peaks in the aliphatic zone (2924 cm\(^{-1}\) and 2875 cm\(^{-1}\)) due to the C-H elongation vibration together with the weak peaks at 1654 cm\(^{-1}\) due to the elongation vibration of C=O bonds and at 1541 cm\(^{-1}\) due to the deformation vibration of N-H bonds and elongation of C-N bonds in the bone powder results from the collagen protein [33-35].

Likewise, the presence of broad bands in the range of 3200-3600 cm\(^{-1}\) result from adsorbed water and carbonate band located at 1450 cm\(^{-1}\) arises due to the presence of CaCO\(_3\) in the buffalo bone [35].

The IR spectrum of the HAp powder obtained from eggshells and sol-gel method except that it contains additional C-H peaks (2924 cm\(^{-1}\) and 2875 cm\(^{-1}\)), which can be linked to the presence of residual organic impurities. The presence of carbonate peak (1450 cm\(^{-1}\) and 870 cm\(^{-1}\)) [35] might imply that it still contains CaCO\(_3\) as impurity in the resulting HAp powder, which will be later confirmed by XRD spectra as shown in Fig. 5.

Fig. 4 shows the XRD patterns of HAp powder synthesized by three different chemical methods: sol-gel method, precipitation method using eggshell and wet chemical precipitation method using CaO and H\(_3\)PO\(_4\) as starting materials.

The XRD patterns show that all the synthesized HAp materials are crystalline. The peaks located at 20 values of 26°, 29°, 32°, 34°, 40°, 47°, 50°, 53° and 64°, correspond to (002), (102), (211), (112), (202), (310), (222), (213) and (004) Miller reflection planes of HAp, respectively (which comply with the JCPDS 09-432 data) [2,5,6,10,12,19, 23,27,30,36].

![Fig. 3](image1.png)

**Fig. 3:** Comparison of the FTIR spectra of bone powder and HAp prepared there from.

![Fig. 4](image2.png)

**Fig. 4:** XRD pattern of HAp powder synthesized by different methods as indicated.

It is quite interesting to note that all the investigated samples have quite similar XRD patterns confirming the presence of the pure apatite phase in
the prepared samples. The absence of peaks corresponding to (024), (021) etc. planes also clearly hinted the absence of phases such as β-tricalcium phosphate [36].

The XRD patterns of the HAp from eggshell powder shows comparatively less impurities than from other methods which confirms that the impurities content in HAp can be reduced by heating the powder at higher temperatures, the same deduction as indicated by the FTIR results.

Fig. 5 shows the XRD pattern of HAp samples obtained from bone powder calcined at two different temperatures, 700 °C and 1000 °C. XRD patterns of both samples are very similar to that obtained by chemical methods. However, the presence of a quite sharp peak located at 20 value of 29° in the HAp prepared by calcination of the bone powder at 700 °C indicates the presence of considerable amount of CaCO₃.

Fig. 5: XRD patterns of HAp obtained from buffalo bone after calcination at 700 °C and 1000 °C.

This peak, however, almost disappears when same HAp powder is heated at 1000 °C, indicating that the decomposition of CaCO₃ takes place at higher temperature. Further, the HAp peaks were found to become sharper with increasing calcination temperature. The results imply the advantages of attaining better purity of the HAp by calcination of the bone powder at higher temperature.

The diffraction peak observed at 20 angle of 32° corresponding to the (211) plane, was chosen for the calculation of the crystallites size, as the former peak can be isolated from other peaks and is relatively sharper than the others. The average crystallite diameter was calculated by using Debye-Scherrer formula as reported elsewhere [12]:

\[ \tau = K\lambda/\beta \cos \theta \]  \hspace{1cm} (i)

where, ‘τ’ is the average crystallite size (in nm), ‘K’ is the shape factor (K = 0.9 for most of the spherical crystallites), ‘λ’ is the wavelength of the X-rays (λ = 1.54056 Å for Cu Kα radiation), ‘β’ is the full width at half maximum (FWHM) (in radian) and ‘θ’ is the Bragg’s diffraction angle. Table 1 compares the crystallite size of HAp particles prepared by various methods.

Table 1: Average crystallite size of HAp prepared by various methods calculated using equation (i).

| S. No. | Methods                                      | 20 (°)* | Crystallite size (nm) |
|--------|----------------------------------------------|--------|-----------------------|
| 1      | Sol-gel                                      | 32.668 | 14.80                 |
| 2      | Precipitation                                | 32.796 | 28.11                 |
| 3      | Eggshell                                     | 32.647 | 17.51                 |
| 4      | Bone (powder calcined at 700 °C)            | 32.748 | 18.06                 |
| 5      | Bone (powder calcined at 1000 °C)           | 32.942 | 29.23                 |

* 20 values corresponding to (211) Miller planes at around 32° for each sample
4. Conclusions

A comparative study on simple, inexpensive, and facile approaches for the synthesis of nano-HAp has been presented. It has been demonstrated that the nano-HAp with reasonably high crystallinity and purity can be prepared in an ordinary senior high school chemistry laboratory. Among the methods presented, the procedure employed on the animal bone offers reliable, most cost effective and greener way of preparing the nano-HAp with high degree of purity and yield.

The FTIR spectra of all HAp samples showed typical peaks centered around 470, 560, 960, 1020 and 1090 cm\(^{-1}\) corresponding to phosphate groups of apatite phase. All the specimens showed the presence of adsorbed water (band 3200-3600 cm\(^{-1}\)) and vibration of OH\(^{-}\) ions (3570 and 1027 cm\(^{-1}\)) in HAp lattices. The presence of peak corresponding to carbonate ions (1426-1490 and 870 cm\(^{-1}\)) revealed the presence of impurities that might not appear on performing the experiment under inert atmosphere.

The XRD results showed that all the synthesized HAp samples were fairly nano-crystalline which also matched the corresponding (JCPDS 09-432) files for apatite crystals with corresponding (002), (102), (211), (112), (202), (310), (222), (213) and (004) lattice planes. The crystallite size of the HAp was in the range 15-30 nm.

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