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Synthesis and spectroscopic investigations of hydroxyapatite using a green chelating agent as template

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\textbf{h}igh\textbf{l}ights

"Hydroxyapatite (HAP) synthesis using green chelating agent as template.
"Sucrose as the chelating agent for the HAP synthesis and compared with commercial.
"Natural sources – pineapple, carrot, sugarcane extracts.
"HAP-purity-uniform morphology-less agglomeration-natural sources than commercial.
"HAP particles-well defined dimensions-reduced size-sugarcane extract.

\textbf{g}raph\textbf{i}cal \textbf{a}b\textbf{str}act

\textbf{a}b\textbf{str}act

Hydroxyapatite [Ca\textsubscript{10}(PO\textsubscript{4}\textsubscript{6})(OH)\textsubscript{2}], HAP particles have been successfully synthesized by a cost-effective, eco-friendly green template method using natural and commercially available sucrose as a chelating agent. The sucrose used in this method has been extracted from various sources, three from natural and one from commercially available sources are exploited in our study to achieve a controlled crystallinity, particle size as well as uniform morphology. Spectral characterizations involving Fourier transform infrared spectroscopy (FT-IR) for the functional group analysis of sucrose and HAP; carbon-13 nuclear magnetic resonance spectroscopy (\textsuperscript{13}C NMR) for the identification of the carbon atoms in sucrose and in HAP; liquid chromatography/mass spectrometry (LC-MS) for the determination of the hydrolyzed products of sucrose; and X-ray diffraction (XRD) techniques for the phase identification of the HAP particles were performed. The morphology of the HAP particles were assessed thoroughly using a scanning electron microscope (SEM) equipped with energy dispersive X-ray analysis (EDAX). The experimental results indicate that the obtained HAP using the natural sucrose as a chelating agent is of phase pure, with a well defined morphology having discrete particles without any agglomeration than the HAP from commercially available sucrose. Further, the reduced particle size can be achieved from the stem sugarcane extract as the source of the chelating agent.

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\textbf{Introduction}

Hydroxyapatite mimics the mineral composition of human bone and teeth and is extensively used as a promising material for various biomedical applications [1]. HAP has also been used as filler for bone defects, coating material for hip prosthesis and nano-compos-
ite particles to treat cancer cells [2–9]. The excellent performance of HAP for various biomedical applications was due to the properties of bioactivity, biocompatibility, solubility and osteoconductivity [10–12]. Hence, it is of central attention to customize its properties such as bioactivity, mechanical strength, solubility and sinterability by controlling its composition, morphology and particle size. Still many methods are attempted to synthesize HAP including hydrothermal [13–15], sol–gel [16–19], microemulsion [20–23] and solid state reactions [24,25]. But all these methods provide a problem associated with the agglomeration of particles with wide range of distribution. Attempts have been made to reduce the agglomeration of particles during the synthesis of HAP and unfortunately they are temporary. In order to overcome this difficulty a chelating agent as template is used which leads the particles without any agglomeration. Our previous publication also proved that glycine acts as a template in the synthesis of HAP particles [26].

Chemical synthesis methods lead to presence of some toxic chemicals which is adsorbed on the surface may have adverse effects in the medical applications. The synthesis protocols for HAP particles involving environmentally mediated materials like plant extract offers numerous benefits of eco-friendliness and compatibility for pharmaceutical as well as biomedical applications. Further, green template synthesis proves to be the best method rather than the chemical and physical method, as it is economical, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals.

We aim to synthesize the HAP particles using a green chelating agent as template under atmospheric pressure, and to investigate their morphology, crystallinity and particle size. This method involves the use of commercially available sucrose and natural sucrose as the chelating agent. Sucrose is a natural substance present in the fruits, vegetables, roots and stem of plants. It is biodegradable, biocompatible, water soluble and is polar in nature. In the acidic solutions, COOH and –OH groups can be generated, which can form stable binding with calcium ions in solutions.

Hence, the present work deals with the green chelating agent as template for the synthesis of HAP powders by using sucrose as chelating agent from natural sources such as pineapple ripe fruit, carrot root and the sugarcane stem. For comparison, HAP powders have also been synthesized using commercially available sucrose. As far as the literature is concerned, there are no such reports claiming the synthesis of HAP by this green chelating agent as template. Hence, we propose this method as a simple and an effective route to produce bulk bioceramic material.

Experimental

Materials and methods

The chemicals calcium chloride dihydrate (CaCl2·2H2O), phosphoric acid (H3PO4), sucrose (C12H22O11), concentrated nitric acid (conc. HNO3), acetonitrile (CH3CN) and aqueous ammonia solution were purchased from Sigma–Aldrich. All the chemicals were Analytical grade and used without any further purification. The aqueous solutions were made by dissolving them in deionized water. In order to get the sucrose from natural sources, the root vegetable carrot (Daucus carota), green ripe fruit pineapple (Ananas comosus) and stem sugarcane (Saccharum officinarum) were purchased from the market at Tamil Nadu, India.

Plant material and preparation of the juice extract

Green ripe pineapple fruit, sugarcane stem and the root carrot vegetable were used to make the extract. After removing the outer layer of the pineapple fruit/carrot vegetable root/sugarcane stem were thoroughly washed in distilled water. The inner fleshy part of about 1 kg was weighed and cut into fine pieces. The extraction of sucrose from the three natural sources was done by water extraction method by using high performance liquid chromatography (HPLC) according to Karkascier et al., protocol [27]. Approximately 10 g of the fine pieces were taken and mixed with 40 mL of deionized water under constant stirring. The obtained juice mixture was filtered through a Whatman No. 1 filter paper (pore size 25 μm). The filtrate was further filtered through 0.6 μm sized filter paper. About 2.5 mL of the filtrate was blended with 7.5 mL of acetonitrile and then the mixture was filtered through a 0.45 μm membrane before injected into HPLC. Simultaneously, the standard sucrose solution of 5–100 μg/mL was prepared and then injected into the HPLC system.

Chromatographic separation of sucrose from various juice extract

The separation of sucrose from the three different natural juice extracts was done by HPLC using Varian Inc., USA equipped with a model 410 Prostar Binary LC with photo diode array (PDA) detector [27]. An amino-bonded carbohydrate column (10 μm, 300 × 4.1 mm) was used with an acetonitrile–water (3:1) mobile phase for isocratic elution at a flow rate of 1.4 mL/min. The eluted sucrose from three different natural juices was compared with the standard sucrose solution. The eluted sucrose was then boiled down to thick syrup and tiny sugar crystals were added to start the crystallization process. Finally, the pure crystals were dried at 40 °C and crushed into powder and then used for the green template synthesis.

Synthesis of HAP

The synthesis of HAP particles prepared using both natural and commercially available sucrose as chelating agent is shown in Scheme 1 (given as an electronic supplementary material). In a typical experimental procedure for the green synthesis of HAP, the pineapple extract powder of 1 wt.% was subsequently dropped into 0.5 M aqueous CaCl2·2H2O solution under the constant stirring followed by the addition of concentrated nitric acid. The above mixture was subjected to continuous stirring for 1 h and to this mixture, 0.3 M aqueous solutions of H3PO4 was also added drop wise with stirring. The molar ratio of Ca2+·PO43– was kept at 1.667 and the pH of the above mixture was adjusted to 9 by using aqueous ammonia solution. A milky white precipitate was obtained and it was kept in an oven at 100 °C for 24 h. The resultant product was washed with ethanol and double distilled water for several times, filtered and dried at 60 °C for about 5 h. The dried powder was calcined and sintered in a muffle furnace at 600 °C for 2 h. The same experimental procedures were carried out for the other two natural sources such as carrot and the sugarcane extract powders. Further, for commercially available sucrose, the same experimental procedure was carried out with a concentration of 1 wt.%.

Characterization

The FT-IR spectral analysis was performed for functional group analysis of the samples by using Nicolet 380 FT-IR spectrophotometer over the range from 4000 to 400 cm−1 with a number of scans 32 and 4 cm−1 resolution. A small amount of commercially available sucrose as well as the extracted sucrose from three different natural sources were blended with KBr and then pressed into a pellet for the analysis. The same procedure was repeated for the as-synthesized HAP powders also.
The $^{13}$C NMR spectra of sucrose (obtained from three different natural sources as well as from commercially available one) and the as-synthesized HAP powders were recorded with a Bruker DPX400 instrument at 100.61 MHz with a 10-mm diameter sample tube at 60 °C, and with AM 360 (90.56 MHz) and 5 mm diameter sample tube at 25 °C. The external standard, placed in a 2 mm (DPX400) or 0.8 mm (AM 360) coaxial inner tube represented DMSO-D$_6$, which provided both reference and ‘lock’. The samples therefore contained no D$_2$O internally and the chemical shifts are reported in the TMS scale.

The hydrolysis products of sucrose were identified by LC–MS. The chromatographic separation of the hydrolyzed product of sucrose was performed using an Agilent Zorax SB-C18 column (50 × 2.1 mm, 5 μm; Polo Alto, CA, USA) with a Phenomenex ODS guard column (4.0 × 3.0 mm, 5 μm; Torrance, CA, USA) at room temperature. Acetonitrile and water (3:1) were used as mobile phase for elution. The flow rate was 1.0 mL/min. The outlet of the column was split and only 0.5 mL/min portion of the column effluent was carried into mass spectrometer.

Mass spectrometric detection was performed on an Agilent G6410 B triple quadrupole mass spectrometer (Agilent Technologies, MA, USA) with an electrospray ionization (ESI) source in the positive ion detection mode. The gas temperature was set at 300 °C and the gas flow was adjusted to 10 L/min. Nitrogen was served as nebulizer (30 psi) and high purity nitrogen served as collision gas (50 psi). Agilent Masshunter B.01.03 software was used for the control of equipment, data acquisition and analysis.

The phase composition, purity and the crystallinity of the as synthesized HAP powders were determined by the X-ray diffractometer (Siemens D500 Spectrometer) with Cu Kα radiation $k=1.5418$ Å generated at $35\text{ kV}$ and $25\text{ mA}$. Data were collected over the 2θ range 20–60° with a step size of 0.010° and a count time of 0.2 s.

Fig. 1. FT-IR spectra of the commercial sucrose as well as the sucrose extracted from various natural sources; (a) commercial sucrose, (b) pineapple extract, (c) carrot extract and (d) sugarcane extract.
The crystalline size of the powder was calculated from the XRD data using the Debye–Scherrer’s equation [28]:

\[ \bar{d} = \frac{K \lambda}{b \cos \theta} \]

where \( \bar{d} \) is the crystal line size (nm); \( K \) the wavelength of monochromatic X-ray beam (nm) (\( K = 1.5418 \) Å for Cu Kα radiation); \( b \) is the full width half maximum (FWHM) for the diffraction peak under consideration; and \( \theta \) the diffraction angle (°). The fraction of crystallinity \( X \) the hydroxyapatite powders was determined [29] from the equation

\[ X = \frac{90.8}{92.29} (100\%) \]

where \( b \) is the FWHM.

The microstructure and chemical constituent changes of the HAP samples were performed by scanning electron microscopy (SEM, Cambridge-S365) equipped with energy-dispersive X-ray microanalysis (EDAX). The EDAX spectra were acquired at an accelerating voltage of 100 keV and the elemental composition was determined using these spectra.

Results and discussion

FT-IR for sucrose

Fig. 1a–d shows the FT-IR spectra for the commercially available sucrose and the sucrose extracted from the three different natural sources. From the figure it is evident that the existence of sucrose in the commercial as well as in the extracts of natural sources well coincided with the previous report by Brizuela et al. [30]. For commercially available sucrose, the strong peaks found at 3561 and 3392 cm\(^{-1}\) are assigned to the ÏˆOH stretching vibration modes. The peaks found at 1239, 1385 and 1632 cm\(^{-1}\) are ascribed to the deformation mode of hydroxyl group. The symmetric C–H vibration peaks were detected at 3012 and 2970 cm\(^{-1}\). The C–H symmetric and anti symmetric stretching vibration peaks were observed at 2941 and 2983 cm\(^{-1}\) for –CH\(_2\) group. Whereas the C–H deformation modes were observed in the following wave numbers: scissoring mode was observed at 1460 cm\(^{-1}\); the peaks found at 1279, 1430 and 1346 cm\(^{-1}\) are attributed to the rocking mode of C–H/CH\(_2\); and the twisting modes of C–H in methylene group were detected at 908 and 849 cm\(^{-1}\), respectively. The C–O stretching bonded to the ÏˆOH group is higher than the C–O–C stretching. The characteristic peaks for C–O stretching were found at 1128 and 989 cm\(^{-1}\), respectively. The C–C stretching vibration modes were found at 1068 and 941 cm\(^{-1}\), respectively. The peaks detected at 869, 682, 582 and 471 cm\(^{-1}\) are ascribed to the deformation modes for O–C–C group, whereas the peak detected at 555 cm\(^{-1}\) is attributed to the deformation mode of O–C–O group. Besides, the peak found at 640 cm\(^{-1}\) is ascribed to the deformation mode of glucofuran ring. The deformation mode of glucopyran ring is observed at 732 cm\(^{-1}\). On examining the FT-IR spectra of sucrose extracted from three different natural sources, the above peaks position are retained as shown in Fig. 1b–d which confirms the presence of sucrose in the three natural extracts.

\(^{13}\)C NMR for sucrose

Fig. 2a–d shows the \(^{13}\)C NMR spectra of sucrose obtained from commercial as well as from three different natural sources like the extracts of pineapple, carrot and sugarcane, respectively. The obtained \(^{13}\)C NMR spectra for the extracted sucrose from three different natural sources and also from commercially available one are listed in Table 1 which confirms the presence of sucrose in the extracts and are well consistent with the earlier reports [31,32]. Especially, the peak found at 92.29 ppm, depicts the anomeric carbon of the pyranose ring from sucrose [33]. The absence of peaks in the range of 98–101 ppm in the obtained spectra reflects the absence of glucose and fructose monosaccharide’s in the extracted sucrose.

Table 1: \(^{13}\)C NMR chemical shifts for sucrose.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>( \delta ) (ppm)</th>
<th>Commercial</th>
<th>Pine apple extract</th>
<th>Carrot extract</th>
<th>Sugarcane extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60.16</td>
<td>60.12</td>
<td>60.20</td>
<td>60.15</td>
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<tr>
<td>2</td>
<td>61.65</td>
<td>61.37</td>
<td>61.47</td>
<td>61.40</td>
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</tr>
<tr>
<td>3</td>
<td>61.41</td>
<td>62.08</td>
<td>62.54</td>
<td>61.92</td>
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<tr>
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<td>66.23</td>
<td>66.97</td>
<td>69.30</td>
<td>69.12</td>
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<td>71.12</td>
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<tr>
<td>7</td>
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<td>76.41</td>
<td>76.43</td>
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<td>81.25</td>
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<td>103.32</td>
<td>103.38</td>
<td>103.50</td>
<td></td>
</tr>
</tbody>
</table>

LC–MS for the hydrolyzed product of sucrose

The quantitative identification of the hydrolyzed products of sucrose was achieved by LC–MS. Fig. 3a–d shows the LC–MS spectra for the hydrolyzed products of sucrose which was obtained from three different natural sources as well as commercial one. From Fig. 3a–d it is clearly evident that the hydrolyzed products of sucrose (obtained from commercial as well as from three different natural sources), were injected directly into the mass spectrometer along with the mobile phase, the analyte yielded predominantly saccharic acid as \([M–2H]^{2–}\) ion at \( m/z = 208.12 \), 2,3,4-tri-hydroxybutyric acid as \([M–H]^{–}\) ion at \( m/z = 135.09 \) and glycolic acid...
acid as [M–H]⁻ ion at m/z = 75.05, respectively. Though in all the cases, the concentration of the hydrolyzed products are found in the order of saccharic acid > 2,3,4-trihydroxybutyric acid > glycolic acid, but its ratios are quite different for each sources of sucrose (Fig. 3a–d). These hydrolyzed products can act as a potential chelating/nucleating agent towards the Ca³⁺PO₄⁻ for the formation of HAP as shown in Scheme 1.

**FT-IR for as-synthesized HAP**

Fig. 4a–d shows the FT-IR spectra of HAP synthesized in the presence of natural and commercially available sucrose as a chelating agent. All the peaks appeared in Fig. 4a–d is attributed to the characteristic peaks of HAP without any trace of impurities as reported in our previous results [34–37]. To account for them, the t₁ phosphate mode is observed at 961 cm⁻¹, whereas the peaks found at 1038 cm⁻¹, 565 cm⁻¹ and 605 cm⁻¹ are attributed to the t₂, t₃ mode of phosphate. The t₄ vibrational mode for phosphate is also observed at 472 cm⁻¹. Besides, the hydroxyl stretching and bending vibration modes are observed at the peak positions 3571 cm⁻¹ and 632 cm⁻¹ respectively. This hydroxyl peak is considered as the confirmative peak for HAP. The adsorption of lattice water was also found at 3443 cm⁻¹ and 1630 cm⁻¹ as stretching and bending modes. Further the highly sensitive FT-IR results indicate that there is no C–H stretching vibrational peaks thus confirming the purity of the HAP sample, i.e., no sucrose moieties are incorporated in the sample as mentioned in the last step of Scheme 1 which may due to the decomposition of hydrolyzed products of sucrose by the process of calcination and sintering. From Fig. 4b–d it is also evident that the above peaks position are retained for the HAP synthesized from natural sources as chelating agent such as pineapple, carrot and the sugarcane extracts.

**¹³C NMR for as-synthesized HAP**

The purity of the as-synthesized HAP derived from three different natural sources of sucrose and also from the commercially available one was evaluated by ¹³C NMR and the corresponding spectra are shown in Fig. 5a–d. The spectra, (Fig. 5a–d) reveal no fingerprint for the existence of carbon which confirms the decomposition of hydrolyzed products of sucrose as shown in Scheme 1 and thus altogether strongly validate the purity and the quality of the as-synthesized HAP from four different sources of sucrose.

**XRD for as-synthesized HAP**

Fig. 6 illustrates the XRD patterns of HAP synthesized in the presence of both commercially available sucrose and sucrose from natural sources as chelating agent. The most important peaks for HAP were observed almost in all the samples with no additional peaks (Fig. 6a–d) and were in agreement with the ICDD card no. 09-432 for stoichiometric HAP. In the case of HAP synthesized using sucrose from the pineapple extract, the intensity of the peaks shown in Fig. 6b seems to be lower when compared to the HAP.
synthesized from commercially available sucrose (Fig. 6a). While changing the sources of sucrose as the carrot extract, the intensity of the peaks again decreases as depicted in Fig. 6c. Upon changing the source as the stem sugarcane extract, the HAP peaks appeared as broad with a lower intensity (Fig. 6d) suggesting the decreased crystallinity of the sample. The above XRD results demonstrate that as we move from the commercially available sucrose to sucrose obtained from natural sources, the intensity of the XRD peaks decrease, and also among the sucrose obtained from various natural sources, the intensity of the peaks decreases from fruit pineapple to stem sugarcane which strongly confirms the decreased crystallinity and crystallite size of the as-synthesized HAP. Further, this fact was also corroborated by the calculated particle size using Scherrer’s equation and is presented in Table 2. For the calculation of crystallinity and crystallite size the plane (0 0 2) was taken since it is sharper and isolated from others. The difference in the crystallinity and crystallite size of the as-synthesized HAP may due to the difference in the concentration of the hydrolyzed products of sucrose as discussed earlier in Fig. 3. From the XRD results, it is concluded that the HAP particle with smaller size can be obtained only by using sugarcane extract rather than the fruit pineapple, root carrot and commercially available sucrose (Table 1).

SEM and EDAX for as-synthesized HAP

Fig. 7 (given as an electronic supplementary material) shows the morphological features of the as-synthesized HAP particles obtained using sucrose from both artificial and various natural sources. The micrograph obtained for the HAP synthesized from commercially available sucrose shown in Fig. 7a reveals the formation of agglomerated particles. Whereas, the HAP obtained from the pineapple extract (Fig. 7b) depicts the formation of discrete HAP particles with uniform cubic like structure. As can be seen in Fig. 7c, the morphology of HAP synthesized using carrot extract resembles capsules like structure homogeneously with discrete in nature. The uniform size distributed discrete particles of HAP having sphere shape without agglomeration is observed when the stem sugarcane extract powder was used as the source of a chelating agent as depicted in Fig. 7d. Obviously the size of the particles seems to be reduced when compared to other natural sources of chelating agent such as fruit pineapple and root carrot extract which was also confirmed by the calculated particle size using Scherrer’s equation as shown in Table 2. The difference in particle size and morphology of the as-synthesized HAP may due to the difference in the concentration of the hydrolyzed products of sucrose as discussed earlier in Fig. 3. The SEM results discussed above portray clearly that the HAP obtained from natural sources are having the properties of well defined morphology with uniform particles size, discrete in nature and less agglomeration than the HAP obtained from artificial sucrose as chelating agent. Also, among the three natural sources of sucrose as a chelating agent, the morphology and size of the HAP particles obtained from stem sugarcane extract is found to be better with respect to reduced particle size and hence it is fixed as an optimum.

Fig. 7a–d, shows the EDAX pattern of the as-synthesized HAP samples using commercially available and natural sucrose as chelating agent. The EDAX pattern depicts clearly the presence of main constituents such as Ca, P and O in the structure of the as-synthesized HAP samples.
Mechanism for the formation of HAP

The hydrolyzation of sucrose and the mechanism for the formation of HAP using sucrose as a chelating agent is shown in Scheme 2 (given as an electronic supplementary material). As shown in the scheme firstly, the H\(^+\) ions provided by the concentrated nitric acid is used as a catalyst in the hydrolysis of sucrose into two fragments such as glucose and fructose. Then the hydrolyzed products of sucrose, namely glucose and fructose can be further oxidized by the concentrated nitric acid. The product for the oxidation of glucose is saccharic acid and for the fructose are glycolic acid and trihydroxybutyric acid respectively. These oxidation products such as saccharic acid, glycolic acid and trihydroxybutyric acid contain the functional groups like –COOH and –OH. These groups involved in the complex formation with the calcium metal ions as Ca-saccharate, Ca-glycolate and Ca-trihydroxybutyrate as depicted in Schemes 1 and 2. However, the chelating ability of –OH groups is weak when compared to the –COOH groups since each Ca\(^{2+}\) ions may attract to the COO\(^-\) in saccharic acid, glycolic acid and trihydroxybutyric acid due to the strong electrostatic attraction. When the phosphate solution is added to the calcium chelating complex, there was an interaction of phosphate ion on Ca-saccharate, Ca-glycolate and Ca-trihydroxybutyrate to nucleate the HAP as HAP-saccharate, HAP-glycolate and HAP-trihydroxybutyrate due to the effect of supersaturation (Schemes 1 and 2). From the above scheme it is evident that sucrose acts as a source of a chelating agent in the formation of HAP with a well defined morphology, reduced size, discrete in nature and less agglomeration.

Conclusion

In summary, the role of the addition of a chelating agent from various sources on the purity, crystallinity and morphology of the HAP particles obtained by the green template synthesis method was investigated. The sucrose extracted from the various natural sources was found to be pure and coincide with the commercially available one as evident from the results of FT-IR and \(^{13}\)C NMR. The LC-MS results reveal the variation in the concentration of the hydrolyzed products of sucrose. The FT-IR and \(^{13}\)C NMR results emphasize that the HAP particles synthesized by this method were found to be pure and free from any organic moieties. The uniform morphology and less agglomerated HAP particles were obtained from the natural sources of sucrose than the commercially available one. The crystallinity, particle size and the morphology of HAP are strongly dependent on the natural sources of chelating agent used, especially the hydrolyzed products of sucrose which was apparent from the LC-MS, XRD and SEM results. The HAP particles of fairly well-defined dimensions with reduced size can be achieved by using only the stem sugarcane extract powder as the natural source of chelating agent. This green template approach towards the synthesis of HAP particles has many advantages such as ease with which the process can be easily scaled up, biodegradability, biocompatibility, economic viability and its use in biomedical applications. The as-synthesized powder can also be used for coating and the further analysis for orthopedic applications will be published later.

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Appendix A. Supplementary material

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References

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A novel green template assisted synthesis of hydroxyapatite nanorods and their spectral characterization

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d Department of Physics, Periyar University, Salem 636 011, Tamilnadu, India

h i g h l i g h t s

" Synthesis of hydroxyapatite (HAP) nanorods by green template assisted method.
" Tartaric acid (TA) as template for the synthesis of HAP nanorods.
" Natural sources of TA-extracts of banana, grape, tamarind fruits.
" HAP rods were nanosized, pure, uniform from the extracts of tamarind than others.
" HAP nanorods by green template-assisted antibacterial activity-preservation of minerals.

a r t i c l e i n f o

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g r a p h i c a l a b s t r a c t

a b s t r a c t

Hydroxyapatite [HAP, Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\)] is the main inorganic component of bone material and is widely used in various biomedical applications due to its excellent bioactivity and biocompatibility. In this paper we have reported the synthesis of hydroxyapatite nanorods by green template method using the extracts of three different natural sources which contain tartaric acid and also from commercially available one. The extracts of banana, grape and tamarind are taken as the sources of tartaric acid. The as-synthesised samples were characterized using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDAX). Also the antibacterial activity of HAP with different concentrations against two pathogen bacteria strains Escherichia coli (E. coli) and Klebsiella (Gram-negative bacteria) were tested. The results show that the particles of all the samples are of nanosized and pure. The crystallinity decreases as changing the sources of tartaric acid from commercial to natural one and also changing the natural sources from banana to tamarind extracts. The formation of nanorods are found in all the samples but the nanorods with uniform size distribution can be obtained only by using the tamarind extract as the source of tartaric acid. Moreover, the as-synthesised HAP nanorods derived from natural sources exhibited a strong antibacterial activity against both E. coli and Klebsiella at a concentration of 100 μg. The HAP nanorods synthesized by this method can act as a potential candidate for various biomedical applications.

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Introduction

Hydroxyapatite is of high relevance in material science, biology and medicine as they constitute the major inorganic phase of human hard tissues like bone and teeth [1-3]. It has been widely studied for the applications of bone replacement, dental defect filling, bone tissue engineering and drug delivery [4-6]. Nanophase HAP can mimic the dimensions of the constituent components of natural tissues which enhance the osteoblast adhesion and resorption of long term tissue engineered implants [7,8]. The properties of HAP including bioactivity, biocompatibility, solubility, sinterability, castability, fracture toughness and absorption can be tailored over wide ranges by controlling the particle size and morphology [1,2,9]. Moreover, the excellent bioactivity of the hydroxyapatite as well as suitable temperature, humidity and nutrition in the human body are also propitious to the adhesion and reproduction of bacteria on its surface, leading to wound infection and disease. Thus, the improvement in the antibacterial activity of HAP is an essential task for the implant to function properly. Besides, bone itself is a composite consisting of HAP nanorods embedded in the collagen matrix [10] and hence the HAP nanorods are desirable when biocompatibility is considered. For these reasons, several researchers have tried to develop HAP nanorods with a special attention.

In recent years, variety of methods have been employed for the synthesis of HAP nanorods which include wet chemical route [11], sol-gel [12], mechanochemical [13], hydrothermal [14], microwave heating [15], microemulsion [16] and template addition [17]. Among them, the template addition is one of the most flexible and convenient method, as it is very effective in producing particles having the size in the range of nanometers together with a minimized degree of agglomeration and a controlled morphology. Our previous publications also proved that the synthesis of hydroxyapatite nanoparticles using glycine and glycine–acrylic acid (GLY-AA) mixed hollow spheres as template material [18,19]. The recent research has been focused on the synthesis of nanoparticles by green template method using ecofriendly natural sources as the template material [20,21]. Tartaric acid is a dihydroxy succinic acid which is soluble in water and is present mainly in fruits like banana, grape and tamarind. Although there are few reports on the synthesis of hydroxyapatite using tartaric acid as template, until now no work has been done using tartaric acid from different natural sources. This motivated us to synthesize hydroxyapatite nanorods by using the extracts of different fruits such as tamarind, grape and banana which contain tartaric acid and also tried the same with commercially available one for comparison. The effect of three different fruit extracts on the purity, crystallinity, morphology, uniformity, size and the distribution of HAP has been investigated through SEM-EDAX, XRD and FTIR. The antibacterial activity is an important study to evaluate the better performance of the materials which is widely used in various technological applications [22,23]. Therefore, in the present work, the antibacterial activities of the as-synthesized hydroxyapatite nanorods have also been studied and the results are discussed.

Experimental

Materials

The chemicals Ca(NO$_3$)$_2$·4H$_2$O (99.99%), H$_3$PO$_4$ (85 wt.% solution in water), tartaric acid and aqueous ammonia were purchased from Sigma-Aldrich. All the chemicals were analar grade and used without any further purification. The aqueous solutions were made by dissolving them in double distilled water. For getting the green template of tartaric acid from three different natural sources, the fresh fruit banana (Musa acuminata), grape (Vitis) and tamarind (Tamarindus indica) were purchased from the market located at Tamilnadu, India.

Preparation of the extract from the fruits

The fresh ripe fruits namely tamarind, grape and banana were used to make the aqueous extract. After removing the outer layer and seeds of each fruit, they were thoroughly washed in double distilled water. The inner fleshy pulp was weighed about 1 kg, which was crushed into 100 ml sterile double distilled water and filtered through Whatman No. 1 filter paper of pore size 25 μm. Then the resultant filtrate was boiled down to thick syrup and dried at 40 °C. The dried product was crushed into powder and then used as the green template for the synthesis of hydroxyapatite.

Synthesis of HAP

The synthesis of HAP nanorods was carried out using both natural and commercially available tartaric acid chelating agent as template. In a typical experimental procedure for the synthesis of HAP, the banana extract powder of 1 wt.% was mixed with 0.5 M aqueous Ca(NO$_3$)$_2$·4H$_2$O solution under the constant stirring. The above mixture was subjected to continuous stirring for 1 h. Subsequently, 0.3 M aqueous solution of H$_3$PO$_4$ was added drop wise to the above mixture with a constant stirring. The molar ratio of Ca$^{2+}$P$^{3-}$ was kept at 1.667 and the pH of the resultant suspension was adjusted to 9 by using aqueous ammonia. A milky white precipitate was obtained and it was kept in an oven at 100 °C for 24 h. The resultant product was washed with ethanol and double distilled water for several times, filtered and dried at 60 °C for about 5 h. The dried powder was calcined and sintered in a muffle furnace at 600 °C for 2 h. The same experimental procedures were carried out for the other two natural sources such as grape and the tamarind extract powders with a concentration of 1 wt.% and also for the commercially available one. The above experiment was repeated for three times inorder to get the reproducibility of the results.

Characterization

The FTIR spectral analysis was performed for functional group identification of the samples by using Shimadzu Japan FTIR-8700 spectrophotometer over the range from 4000 to 400 cm$^{-1}$ with a number of scans 32 and 4 cm$^{-1}$ resolution. For this, a small amount of the as-synthesized HAP powders were mixed with KBr homogeneously and converted into pellets under a pressure of 8 ton and the spectra (wave number Vs% transmittance) were taken thereafter.

The phase composition, purity and the crystallinity of the as synthesized HAP powders were determined by the X-ray diffractometer (Siemens D500 Spectrometer), with Cu Kα radiation generated at 35 kV and 25 mA. Data were collected over the 2θ range of 20–60° with a step size of 0.010° and a count time of 0.2 s. The crystallite size of the powder was calculated from the XRD data using the Debye–Scherrer’s equation [18–24]:

$$\delta = \frac{K \lambda}{B \cos \theta}$$

where K is the broadening constant chosen as 0.9, θ is the crystallite size (nm); k the wavelength of monochromatic X-ray beam (nm) (k = 1.5418 Å for Cu Kα radiation); b is the full width half maximum (FWHM) for the diffraction peak under consideration; and h the diffraction angle (°). The fraction of crystallinity X of the HAP nanoparticles was determined [18,19,25] from the following equation
where $b$ is the FWHM.

The morphological characteristics and the chemical constituents of the HAP samples were analyzed by scanning electron microscopy (SEM, Cambridge-S365) equipped with energy-dispersive X-ray microanalysis. The instrument Philips 501 scanning electron microscope equipped with EDAX was used for the determination of microstructural analysis and elemental composition. The EDAX spectra are acquired at an accelerating voltage of 100 keV.

Antibacterial activity studies

The antibacterial activity of the as-synthesized HAP has been investigated against both E. coli and Klebsiella as the model Gram-negative bacteria by the agar well diffusion method [26]. The inoculums of the two microorganisms such as E. coli and Klebsiella were prepared from fresh overnight broth cultures (Tripton soy broth with 0.6% yeast extract) that were incubated at 37 °C. The resulting broth cultures were used for the diffusion tests.

The agar diffusion test was performed at Muller–Hinton agar and it was carried out by pouring agar into petri dishes to form 4 mm thick layers and further adding 2 ml dense inoculums of the tested microorganisms in order to obtain semi confluent growth. Petri plates were left for 10 min to dry in air and then the HAP samples to be tested with different volumes were impregnated into the well against the inoculated microorganisms on the agar surface and were incubated for 24 h at 37 °C. The volumes of HAP used in the test were 25, 50, 75 and 100 μl, respectively. The different volumes of HAP samples were taken from 1 wt.% concentration of HAP which was prepared by dissolving 0.2 g of HAP samples in 2 ml of dimethyl sulphoxide (DMSO). Finally, the inhibition zone (mm) was monitored by measuring the width around the well.

Results and discussion

FTIR

Fig. 1a–d shows the FTIR spectra of HAP samples synthesized in the presence of natural and commercially available tartaric acid chelating agent as a green template. The results of FTIR patterns presented in Fig. 1a–d confirm the formation of hydroxyapatite without any trace of other calcium phosphate phases [10,18,19,27–29]. To account for them, the $\nu_1$ phosphate mode is observed at 954 cm$^{-1}$, whereas the peak pairs found at 1086 and 1045 cm$^{-1}$ and 605 and 563 cm$^{-1}$ are attributed to the $\nu_3$ and $\nu_4$ modes of phosphate respectively. The $\nu_2$ vibrational mode of phosphate is also evidenced at 472 cm$^{-1}$. Besides, the hydroxyl stretching vibrational mode is observed at the peak position 3572 cm$^{-1}$ and it is considered as the confirmative peak for HAP. The absorption of lattice water was also found at 3447 and 1630 cm$^{-1}$ as stretching and bending modes. Further the highly sensitive FTIR results indicate that there is no C–H stretching vibrational peaks thus confirming the purity of the HAP sample i.e., no tartaric acid moiety is incorporated in the sample. From Fig. 1b–d, it is also evi-

![Fig. 1](image1.png)

![Fig. 2](image2.png)
dent that the above peaks are retained for the HAP sample synthesized from natural sources of tartaric acid chelating agent such as tamarind, grape and the banana extracts.

XRD

Fig. 2 illustrates the XRD patterns of HAP synthesized using tartaric acid as green template which is obtained from both commercial and different fruit sources. As can be seen in Fig. 2a–d, the most important peaks of HAP were observed in all the samples with no additional peaks correspond to other calcium phosphates. The obtained results were in concurrence with the ICDD card No. 09-432 for stoichiometric HAP. For the HAP sample synthesized using commercial tartaric acid as the template (Fig. 2a), the intensity of the peaks seems to be higher when compared to the intensity of the HAP peaks for the samples derived from tartaric acid obtained from the extracts of the three different natural fruit sources (Fig. 2b–d). Among the three different natural fruit sources of tartaric acid, for the HAP synthesized using banana extract, the intensity of the peaks shown in Fig. 2b is higher when compared to the HAP synthesized from grape extract as depicted in Fig. 2c. Upon further changing the source of tartaric acid as the tamarind extract, the peaks for HAP appeared as broader with a lower intensity (Fig. 2d) thus, strongly suggesting the decreased crystallinity and particle size of the sample. From the XRD results it is evident that as the intensity of the HAP peaks decreases, the peak broadening nature increases which altogether reflects the effect of decreased crystallinity as well as the particle size. The above facts were confirmed by the particle size calculated by using Scherrer’s equation and the obtained values are presented in Table 1. The diffraction plane (0 0 2) was taken for the calculation of crystallinity and crystallite size since it is sharper and isolated from others. By

![Figure 2](image1.png)

![Figure 3](image2.png)

![Figure 4](image3.png)

Table 1: Structural properties of the as-synthesised powders.

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>Plane</th>
<th>Line width (FWHM)</th>
<th>Fractional crystallinity (Xc)</th>
<th>Crystallite size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial tartaric acid</td>
<td>002</td>
<td>0.142</td>
<td>4.8</td>
<td>62</td>
</tr>
<tr>
<td>Banana extract</td>
<td>002</td>
<td>0.154</td>
<td>3.8</td>
<td>57</td>
</tr>
<tr>
<td>Grapes extract</td>
<td>002</td>
<td>0.166</td>
<td>3.0</td>
<td>53</td>
</tr>
<tr>
<td>Tamarind extract</td>
<td>002</td>
<td>0.180</td>
<td>2.4</td>
<td>49</td>
</tr>
</tbody>
</table>

Fig. 3. SEM micrographs and EDAX patterns of the HAP nanorods using tartaric acid as template from commercially available and three different natural fruit sources: (a and a₁) commercial tartaric acid, (b and b₁) banana extract, (c and c₁) grapes extract and (d and d₁) tamarind extract.

Fig. 4. Antibacterial activities of the HAP nanorods against the gram negative bacteria (a) E. coli and (b) Klebsiella respectively.
consolidating the XRD results, it is emphasized that the HAP nanoparticles with smaller particle size and lower crystallinity can be obtained only by using tamarind extract as the source of tartaric acid rather than the extracts of grape, banana and also commercially available one (Table 1).

SEM and EDAX analysis

Fig. 3 shows the morphological features of the as-synthesised HAP samples using tartaric acid as template obtained from both commercial and three different natural fruit sources. The synthesis process at each sources of tartaric acid was repeated for three times in order to check the reproducibility of the results. But the morphological images of the obtained nanostructure from the four different sources of tartaric acid are presented only for one trial and are shown in Fig. 3. The shape, size, distribution, aspect ratio and standard deviation of the obtained nanostructures are discussed in detail as follows. In all the micrographs the formation of nanorods was evident as shown in Fig. 3a–d. However, the uniformity, size and the distribution of the nanoparticles are diverse while changing the sources of tartaric acid from commercial to the naturally available one. The micrograph for the HAP nanopowders synthesised from commercially available tartaric acid as shown in Fig. 3a depicts the formation of nanorods but it is not uniform throughout. Though discrete nanorods were obtained, its distribution seems to be in a wide range. The size of the nanorods was ranging from 105.12–200.82 nm in length and 32.00–95.12 nm in diameter and the aspect ratio ranges from 1.67–4.12. The mean length, diameter, aspect ratio and standard deviation of the obtained nanorods are found to be 149.49 nm, 57.52 nm, 2.6 and 0.84, respectively. Whereas, for the HAP synthesised from the extract of banana (Fig. 3b), the formation of few discrete nanorods with majority agglomerated nanorods in the form of ice cubes were obtained. The length, width and aspect ratio of the very few as-formed discrete nanorods are ranged from 85.92–192.18 nm, 24.21–40.12 nm and 2.43–6.77, respectively. The mean length, width, aspect ratio and the standard deviation of the few discrete nanorods are found to be 142.86 nm, 31.77 nm, 4.49 and 1.58 respectively. As can be seen in Fig. 3c, the morphology of the HAP synthesized using grape extract also resembles rod like structure but its formation is not uniform throughout. The length of the nanorods is ranged from 110.36–276.34 nm, width from 22.26–86.37 nm and aspect ratio ranges from 2.86–8.97. The mean length, width, aspect ratio and standard deviation of the obtained nanorods are found to be 207.38 nm, 33.53 nm, 6.18 and 1.82 respectively. Obviously as can be seen in Fig. 3d, the formation of nanorods is quite uniform throughout and the size of the nanorods seems to be reduced for the HAP synthesised using the extract of tamarind when compared to the other natural sources of template such as the extract of banana, grape and also commercially available one. The length, width and aspect ratio of the nanorods are ranging from 162.16–298.54 nm, 26.86–34.81 nm and 5.38–9.65, respectively. The mean length, width, aspect ratio and standard deviation of the obtained nanorods are likely to be 230.33 nm, 30.12 nm, 7.65 and 1.18, respectively. The uniform size of the distributed HAP nanorods may be due to the presence of enough concentration of tartaric acid in the extract of tamarind to react with the available Ca\(^{2+}\) ions to form calcium tartrate complex. This calcium tartrate complex further reacts with PO\(_4\)\(^{3-}\) ions to form uniform HAP nanorods. In case of the extracts of grape and banana, the concentration of tartaric acid is low to react with the Ca\(^{2+}\) and then with PO\(_4\)\(^{3-}\) ions and hence the agglomerated nanorods

![Photographs of antibacterial activities of the HAP nanorods against the gram negative bacteria E. coli](image)

(a) HAP nanorods from commercial tartaric acid, (b) HAP nanorods from the extracts of banana, (c) HAP nanorods from the extracts of grapes and (d) HAP nanorods from the extracts of tamarind, respectively.
Antibacterial activity of the as-synthesized HAP nanorods

The antibacterial activity of the as-synthesized HAP nanorods at four different volumes such as 25, 50, 75 and 100 μl were measured against the two gram negative bacteria like E. coli and Klebsiella. The results are presented in Fig. 4a and b and its photographs are shown in Figs. 5 and 6. E. coli and Klebsiella are the general bacteria that are found in the contaminated wound. It was found that the HAP nanorods exhibited an inhibition zone and for the one synthesized from commercial tartaric acid, the measured inhibition zone was found to be 1 mm for both the E. coli and Klebsiella (Figs. 4a and b and 5a and 6a). Upon increasing the concentration of HAP, the measured inhibition zone for E. coli and Klebsiella were found to be 2 and 3 mm for 50 μl, 5 and 5 mm for 75 μl and 7 and 6 mm for 100 μl, respectively (Figs. 4a and b 5a and 6a). Whereas the antibacterial activity for the HAP nanorods derived from three different green fruit extracts like banana, grapes and tamarind are found to be higher than that of the commercially available tartaric acid (Figs. 4a and b and 5b–d and 6b–d). This may be due to the presence of minerals such as Mg2+, Na+ and Zn2+ in the as-synthesized HAP nanorods which may come from the extract of natural fruit sources. The presence of these minerals was corroborated by the EDAX analysis as discussed earlier (Fig. 3b–d). Among the three natural fruit extracts used, the antibacterial activity of HAP derived from tamarind extract at 100 μl is found to be higher (14 mm, 12 mm) than the extracts of grape (13 mm, 11 mm) and banana (11 mm, 9 mm) for both E. coli and Klebsiella (Figs. 4a and b and 5b–d and 6b–d). Similarly even at lower concentration such as 25 μl, 50 μl and at 75 μl, the antibacterial activity of HAP nanorods derived from the extracts

Table 2
Composition of minerals in natural fruit extracts [30].

<table>
<thead>
<tr>
<th>Fruit sources</th>
<th>Minerals (mg)</th>
<th>Mg</th>
<th>Na</th>
<th>Zn</th>
<th>Vitamin E (contains Zn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial tartaric</td>
<td>–</td>
<td>–</td>
<td>0.15</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Extracts of banana</td>
<td>27.0</td>
<td>3.03</td>
<td>0.07</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Extracts of grapes</td>
<td>92.0</td>
<td>28.0</td>
<td>0.10</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Extracts of tamarind</td>
<td>92.0</td>
<td>28.0</td>
<td>0.10</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>
of tamarind is higher than that of the extracts of grapes, banana and the commercially available tartaric acid. According to the United State Department of Agriculture (USDA) data base [30], the concentration of Mg\(^{2+}\) and Na\(^{+}\) ions in 100 g of fruit extracts of tamarind are higher than their concentration in other two fruit extracts as shown in Table 2. Natural bone contains different trace elements such as Sr\(^{2+}\), Ba\(^{2+}\), Pb\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), F\(^{-}\) and CO\(^{2-}\) which play an important role in the biological performances of bone [31,32]. Mg has its own significance in the calcification process and on bone fragility, indirect influence on mineral metabolism and also has influence on HAP formation and growth. Also, magnesium ions have been found to inhibit growth of the (0 0 1) face of HAP crystals [33]. The Mg-substituted HAP materials are expected to have an excellent biocompatibility and biological properties. Mg-HAP ceramics have been proposed for use in orthopaedic and dental applications [34]. Further, Zn is an essential trace element for promoting osteoplastic cell proliferation and differentiation. The Zn\(^{2+}\) ion is involved in many metalloenzymes and proteins, including alkaline phosphates [35]. Zinc is a trace mineral and has been used in the healing of wounds because it can increase the speed of healing. According to the University of Maryland Medical Center, zinc is also important in keeping the immune system healthy, which is vital for protecting the body against bacteria and infections. Zinc may be effective in preventing acne formation and zinc like vitamin E, is also an antioxidant vitamin. In our earlier study, we have reported the synthesis of minerals (Sr, Mg and Zn) substituted nanohydroxyapatite via amino acid soft solution freezing method [35] to mimic the composition of natural bone and also to enhance the biological function of bone. The enhanced antibacterial activity of HAP nanorods from tamarind extract was attributed to the presence of higher concentration of Mg\(^{2+}\), Na\(^{+}\) and essential Zn\(^{2+}\) than other two fruit sources (Table 2). Singh et al. [36] reported that the tamarind extract is used as anti-viral and antifungal agents. From these results it is clearly demonstrated that in all the HAP samples as the concentration is increased from 25 \(\text{mL}\) to 100 \(\text{mL}\), the inhibition zone for both E. coli and Klebsiella was also dramatically increased which reflects the good antibacterial activity against both the bacteria. Since minerals are the constituents of bone, its presence even in trace level may play a vital role in enhancing the antibacterial activity of HAP.

Conclusions

In summary, hydroxyapatite nanorods with excellent antibacterial properties have been successfully synthesized using the green template method. Tartaric acid is used as green template for the overall synthesis of HAP which has been obtained from the extracts of fresh fruits such as banana, grapes and tamarind, respectively. The synthesis of HAP using commercially available tartaric acid was also carried out for comparison purpose. The phase pure HAP nanorods without any impurities have been found from the FTIR and XRD results. When changing the sources of tartaric acid from commercial to natural, the crystallinity and crystallite size were decreased. Among the three different natural fruit sources, the HAP nanorods obtained from the extracts of tamarind possesses reduced crystallinity and crystallite size when compared to the HAP nanorods from the extracts of grapes and banana. SEM images showed that even though the nanorods were formed in all the cases, the uniform size distribution was obtained only by using the tamarind extract as the green template when compared to other fruit sources. The antibacterial results reveal that the as-synthesised HAP nanorods exhibited a strong antibacterial activity against both the Gram-negative bacteria of E. coli and Klebsiella. The HAP nanorods synthesized by this green template method can be used as an impending material for various biomedical applications.

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