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Characterization and Antibacterial Activity of Hydroxyapatite nanoparticles

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Abstract: Nanoparticles are tiny materials with different properties as compared to their bulk material of the same composition. Their particular physiochemical and biochemical properties make them significant for commercialization. Nano Hydroxyapatite (HAP) is an ideal biomaterial that has the potential to facilitate strong bone conduction. It can hence be used for bone grafting, dentistry and for blocking cancerous cell proliferation. In this study, commercial hydroxyapatite nanoparticles were characterized using particle size, Fourier Transform Infrared Spectroscopy and Scanning Electron Microscopy. Their antimicrobial activities were also tested against nine standard MTCC cultures and also against normal skin flora by administering a specific concentration of HAP nanoparticles to check the zone of inhibition using well diffusion method.
Key words: Hydroxyapatite nanoparticles, particle size analyses, SEM, antibacterial activity.

Introduction

Nanotechnology is currently an area of intense scientific research. Nanoparticles act as effective bridges between their respective bulk materials and their atomic or molecular structures. The properties of materials change as their size approaches the nanoscales as the number of atoms at the surface of a material become significantly high. Due to this, these nanoparticles have characteristic optical properties as they are small enough to confine their electrons and produce quantum effects, for *e.g.,* gold nanoparticles appear deep red to black in solution due to high surface area to volume ratio that reduces the melting temperature of these nanoparticles[1].

Hydroxyapatite (HAP) has a wide range of applications in the several areas such as semiconductor and electronic industries, advanced composite material (e.g. carbon nanotube polymers), and paint industries and in the biomedical arena. In the pharmaceutical industry, these nanoparticles have been employed to improve the activity and immobilization of catalysts by providing large interfaces. This imparts high catalytic activity and enhances the adsorption capability in various related fields[2]. Application of pure HAP is very limited due to its brittle nature. Therefore it has been modified by polymerization to utilize it as artificial bone substitute since natural bones consist of nanosized, needle shaped HAP crystals[3, 4]. It is an ideal biomaterial as it supports the proliferation of the normal bone cells (osteoblasts) by causing programmed cell death in osteosarcoma cells. This helps in repairing the bone defects caused by osteoporotic fractures[5]. Toxicity studies on rabbits have revealed that HAP nanoparticles have no accumulative noxious effect on them. However, it is suggested to be safe when the HA-sol is applied intravenously as a drug carrier in small dosages as compared to medium lethal doses[6]. HAP nanoparticles have the potential to be used as anticancer agents as they prevent the adhesion of cancerous cells thereby blocking proliferation. They are found to restrain the growth of MG C80-3 cells, osteosarcoma OS-732 cells, esophageal Ec-109 cells and ten other cells *in vitro*[7]. Cerium has certain antibacterial abilities therefore cerium substituted HAP particles (Ce-HAP) result in increase of solubility that

in turn enhances the biodegradability and antibacterial property[8,9].Furthermore these nanoparticles have been employed as soil additives for *in situ* remediation of metal contaminated soil. Exchangeable metals like sodium, potassium, calcium and magnesium show significant decrease with increasing HAP application. Plant growth gets partly restored with 0.5% and 1% HAP treated soils whereas it gets inhibited at 5% concentration[10].

In the present study characterization of HAP nanoparticles was carried out using particle size, zeta potential and electrophoretic mobility, FTIR and SEM. The nanoparticles were also assessed for their antibacterial activity against nine standard MTCC isolates and four normal skin flora using Kirby Bauer well diffusion method.

Materials and Method

Chemicals and media:

All media components were procured from Hi media, commercially available HAP nanoparticles were kindly donated by NAL-CSIR, Bangalore.

Instruments used:

Fourier Transform Infrared Spectrophotometer was from BRUKER, Optics, Germany. Particle size and zeta potential analyser was from Horiba Scientific SZ 100 and was equipped with Windows [Z type] version 2.00 software (United States). Scanning Electron Microscopic analysis was done using Carl Zeiss EVO 18 Research model.

Microbial cultures:

The microbial cultures used in the study were 441- *Bacillus subtilis,* 3160-*Staphylococcus aureus,* 9493-*Proteus mirabilis,* 443-*E coli, 7298-Serratia marcescens, 451- Vibrio parahaemolyticus.* The following strains of skin flora were also used, *viz., Vibrio spp., Pseudomonas spp., Proteus spp., Staphylococcus spp.*

Characterization:

Particle Size and Zeta Potential analysis:

Particle size, zeta potential, polydispersity index and electrophoretic mobility were recorded for 1/100 diluted HAP nanoparticles after sonication using a water bath sonicator for 15 minutes. The scattering angle, the dispersion medium viscosity and the temperature for the analysis were taken to be 90˚, 1.996 mPa·s and 25.2˚C respectively. The count rate of the hydroxyapatite nanoparticle was 45 kCPS.

FT-IR:

Fourier Transform Infrared Spectrophotometery was done to identify its specific functional groups. It is a sensitive method that relies on the fact that the particular sample absorbs light in the infra-red region of the electromagnetic spectrum. The absorption corresponds specifically to the bonds present in the HAP nanoparticle. The IR reflection spectra of HAP nanoparticles were recorded with attenuated total reflection technique and measured at room temperature using FT-IR spectrometer in the spectral range of 4000–5000cm^{-1} for functional group analysis[11].

Scanning Electron Microscopy:

The structural morphology of the nanoparticles was studied with a Scanning Electron microscope.

Antibacterial activity:

Isolation and identification of skin flora:

To evaluate the toxicity of the nanoparticles against skin associated bacteria, the skin flora was collected using sterile cotton swabs and these were subsequently plated on nutrient agar plates. Incubation of the plates was done at 37˚C for 24 hours. Morphological characteristics of the isolated colonies were recorded

using gram staining. These colonies were then sub-cultured onto separate agar slants and the genus of the organisms was identified by performing different biochemical tests[8, 11, 12].

Activity against skin flora:

Kirby Bauer well diffusion method was used for testing the antimicrobial activity of the isolated skin flora[1]. Muller Hilton agar plates were prepared and the isolated organisms were swabbed onto them and incubated at 37°C for 24 hours. Different dilutions of the nanoparticles were prepared by dissolving 100 µg, 500 µg and 1000 µg in 1ml of distilled water. A sterile well borer was used to punch wells onto the plates and 100µl of the dilutions were added into different wells. Selective antibiotic discs were used as positive control. The plates were incubated at 37°C overnight and were then checked for zones of inhibition[11, 13].

Table1: Antibiotic discs used as positive control.

Sl. No.	Microorganism	Positive control (antibiotic disc)
1	*Proteus* spp.	Streptomycin
2	*Bacillus* spp.	Penicillin
3	*Staphylococcus* spp.	Penicillin
4	*Pseudomonas* spp.	Ciprofloxacin

Activity against MTCC cultures:

Kirby Bauer well diffusion method was used for testing the antimicrobial activity on the six cultures as mentioned above. The test cultures were inoculated into nutrient broth and incubated at 37˚C for 24 hours and then swabbed onto Muller Hinton Agar plates using sterile swabs[13]. The concentrations of the nanoparticles prepared were same as above. 100µl of each of the sample were added to the different wells. Selective antibiotic discs were used as positive controls. The plates were incubated overnight at 37˚C and observed for zones of inhibition[8,11,12].

Table2: Antibiotic discs used as positive control.

Sl. No.	Microorganism	Positive control (antibiotic disc)
1	*Escherichia coli*	Ciprofloxacin
2	*Bacillus subtilis*	Penicillin
3	*Staphylococcus aureus*	Penicillin
4	*Proteus mirabilis*	Streptomycin
5	*Serratiamarcescens*	Chloramphenicol
6	*Vibrio parahaemolyticus*	Ciprofloxacin

Results and Discussion:

Characterization of HAP nanoparticles:

The mean diameter of the particles was found to be 38.4±2.3nm. This corresponds to the results obtained by Chandrasekar *et al.*[4]**.** The polydispersity index was recorded to be 2.0. The particles may thus be polydisperse in nature. **Figure1** represents the results of particle size analysis.

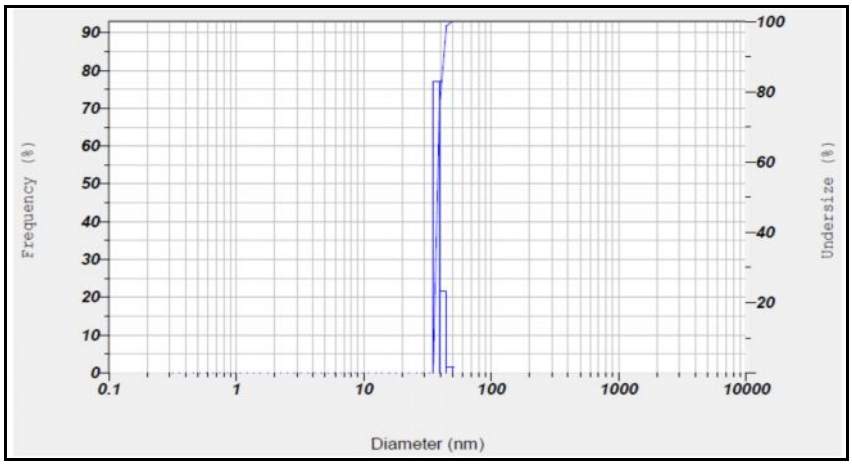


Fig 1: Particle size analysis of hydroxyapatite nanoparticles

The zeta potential and electrophoretic mobility was found to be -55.0 mV and -0.000117 cm^2/Vs respectively as depicted in **Figure 2**.

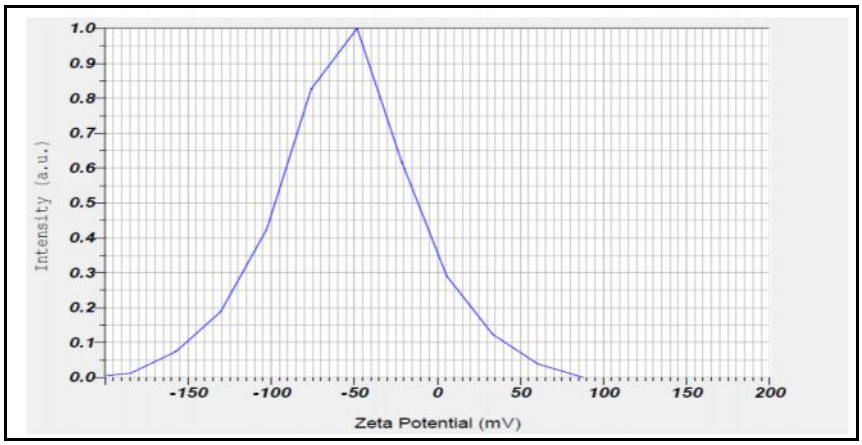


Fig 2: Zeta potential analysis of HAP nanoparticles

Fourier transform infrared spectroscopy (FTIR) was used for the characterization of the nanoparticles[11]. The functional groups present and recorded in the spectra were hydroxyl group, carbonyl, carboxylic acid and phosphate groups. The frequencies of the resultant peaks obtained were compared with the standard FTIR chart. **Figure 3** depicts the peaks obtained for the nanoparticle. Previous reports by Chandrasekar *et al.* depicted the stretching vibrations and the functional groups corresponding to the present values[11].

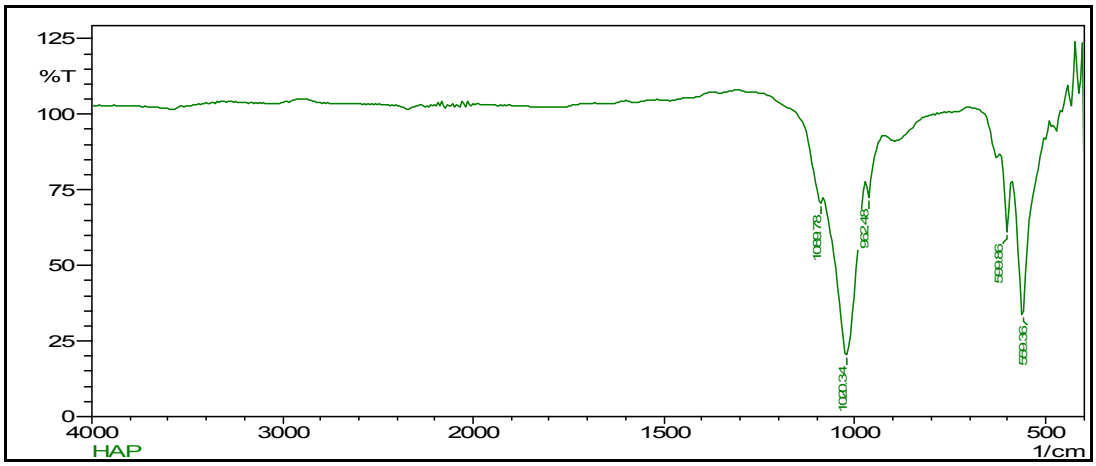


Fig 3: FT-IR analysis of HAP nanoparticles

Scanning Electron Microscopy was done to know the morphological details. The HAP nanoparticles were highly agglomerated. The morphology is given in **Figure 4.**

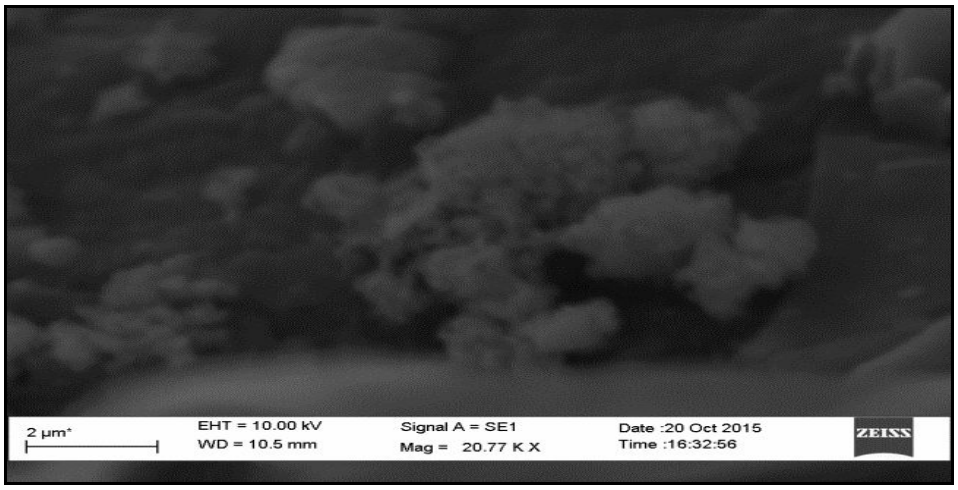


Fig4: Scanning Electron Microscope image of HAP nanoparticles

The standard EDX spectra of the nanoparticles confirmed the presence of O, P, Ca and also showed the presence of traces of carbon as shown in Fig 5a and 5b[10].

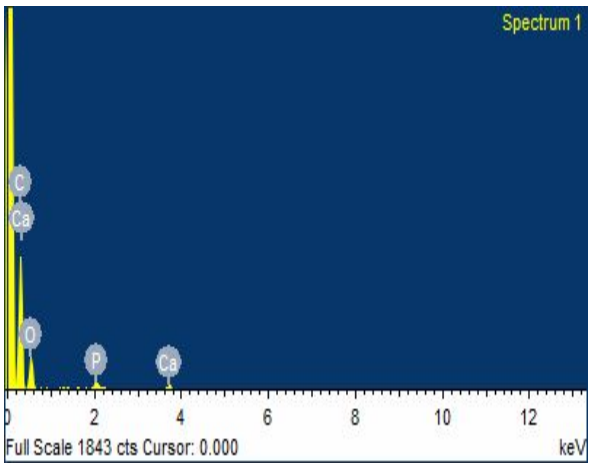


Fig 5a: EDX spectra of HAP nanoparticles

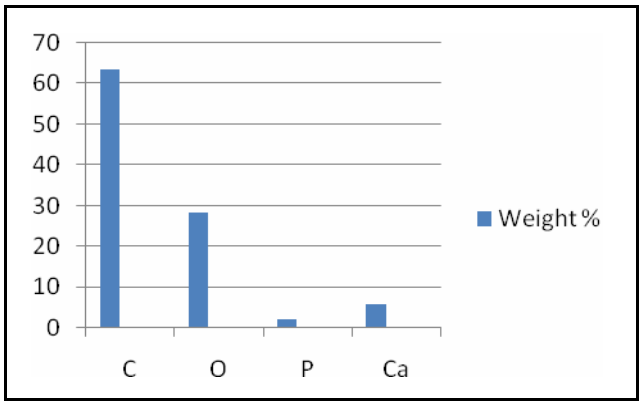


Fig 5b: Weight % EDX spectrum of HAP nanoparticles

Antibacterial activity

The antibacterial activity was assessed against six MTCC cultures and four skin flora by Kirby Bauer well diffusion method. The results showed a slight zone of inhibition in case of *Staphylococcus aureus*(3160). No substantial zones were observed for the other bacterial cultures. The nanoparticles were dispersed in distilled water for the antibacterial activity. However higher concentrations of the HAP nanoparticles might show greater zones. An antibacterial study using *Escherichia coli, Staphylococcus aureus,* and *Lactobacillus* with HAP has shown *Lactobacillus* to have high zone of inhibition[14, 15]. All the CeHAP (cerium substituted) nanoparticles have a greater antibacterial ratio than HAP nanoparticle, and the antibacterial activity of CeHAP nanoparticle gets higher with the increase of cerium substitution, representing that the antibacterial property is enhanced after Ce partially substitutes for Ca^{++} in the structure of HAP. It may be due to the greater solubility of CeHAP compared to HAP, and more Ce^{3+} are released to inhibit the existence of tested bacteria as the increase of cerium in HAP[8, 11, 12].

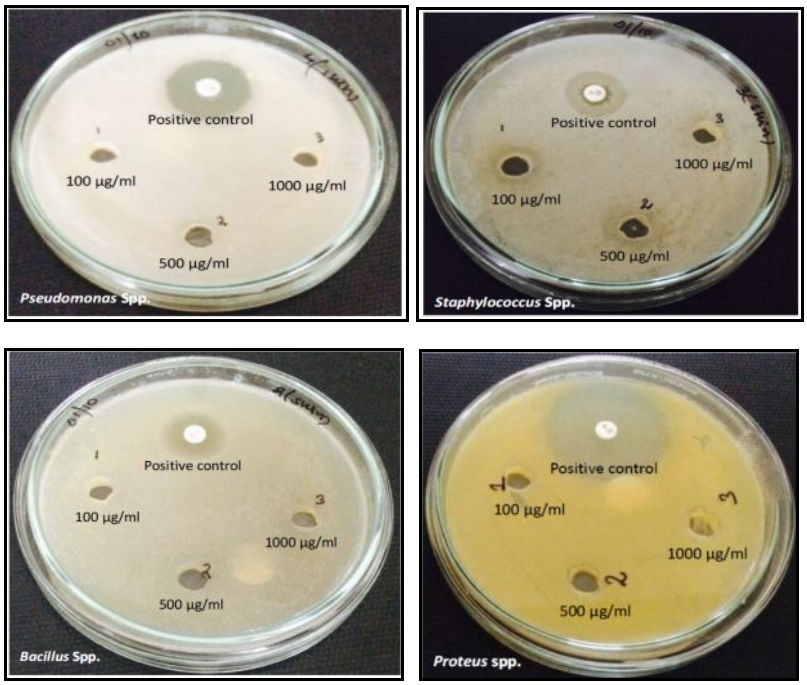
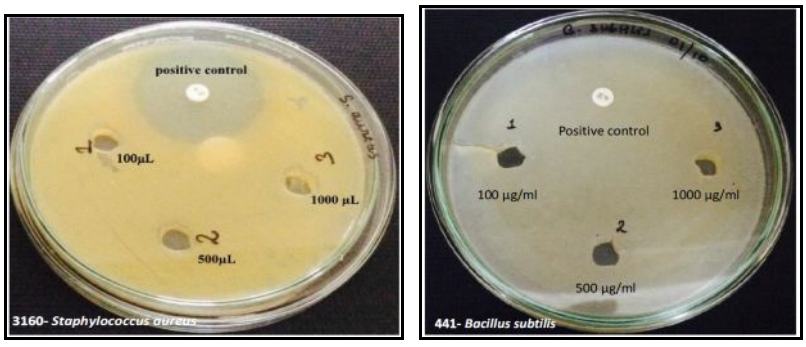


Fig7(a): Antibacterial activity of HAP against isolated skin flora



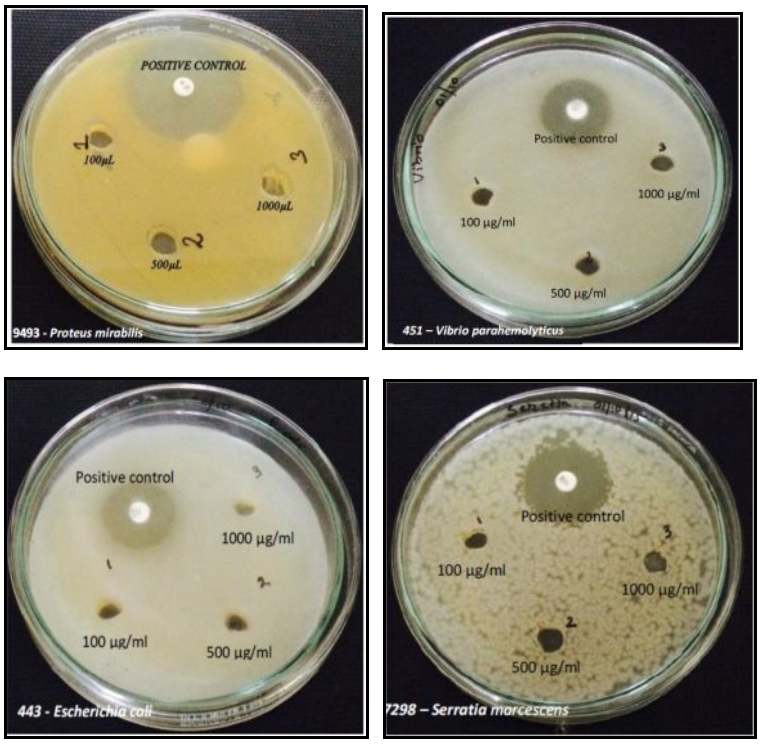


Fig 7(b):Antibacterial activity of HAP against bacterial cultures

Conclusion:

Commercial HAP nanoparticles were characterised using Particle size analyser, FT-IR, and Scanning Electron Microscopy. The nanoparticle dispersion in distilled water with various concentrations were tested against MTCC cultures and isolated skin flora for evaluating its antibacterial activity. Since there was no substantial activity observed for water dispersion of the nanoparticle, it can be regarded as a potentially harmless agent that could be used in different industries like cosmetics and medicine.

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