Materials Science and Engineering C 73 (2017) 8–14

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Mechanistic investigation on microbial toxicity of nano hydroxyapatite on implant associated pathogens

K. Baskar [a], T. Anusuya [b], G. Devanand Venkatasubbu [b,*]

[a] Department of Biotechnology, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India
[b] Department of Nanotechnology, SRM University, Kattankulathur, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form 7 November 2016
Accepted 13 December 2016
Available online 15 December 2016

Keywords:
Biomaterial
Osteomyelitis
Implant
Lactate dehydrogenase
Cytotoxicity

ABSTRACT

The use of atomic scale inorganic nanoparticles (NPs) to fight against pathogenic microorganisms is a recent trend in biomedical area which overcomes the limitations of organic compounds in terms of stability, shelf life and bioactivity. One such Calcium phosphate based biomaterial is hydroxyapatite (HA), considered as potential bioactive compound with excellent biocompatibility, osteointegrity and biodegradability. Osteomyelitis, the implant associated infection, is the major problem worldwide responsible for the majority of implant failure cases. Since HA is used as a coating material of implants, only few reports were available on its antimicrobial activity and cytotoxicity whereas no reports on its possible antimicrobial mechanism. In this present study, the HA-NPs were synthesized by wet chemical precipitation and were characterized using X-ray diffraction (XRD), Transmission Electron Microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). The synthesized HA-NPs were evaluated for antimicrobial activity against implant associated bacterial pathogens. The study also explores the mechanistic action of HA-NPs in killing of bacteria by determining the reactive oxygen species (ROS) generation, DNA fragmentation, Lactate dehydrogenase (LDH) leakage and cellular interaction. In addition the cytotoxicity of HA-NPs was determined by MTT assay and Fluorescence Microscopic analysis. The results revealed that, the synthesized HA-NPs showed good antibacterial activity for tested bacterial species and the possible antibacterial mechanism were due to the lack of membrane integrity and cytotoxic studies shows the concentration dependent changes in cell viability.

1. Introduction

In the last few years, inorganic nanobiomaterials have received considerable attention in biomedical area than organic materials due to their unique chemical, physical and biological properties such as stability, high surface area, long shelf life and anti-microbial applications. Some of the inorganic materials that have been tested for their antimicrobial properties are TiO_2, ZnO, MgO, CaO, CuO, Al_2O_3, Ag_2O and CeO_2 [1]. Calcium orthophosphate based synthetic hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), the major mineral component of human bone and other calcified tissues and classified as a bioactive material that supports bone growth and osteointegration when used in orthopedic, dentistry and maxillofacial applications [2]. HA is not only the main component of hard tissues, but also has excellent affinity to biomolecules such as proteins [3] and used as implant material both in bulk porous form, for filling in or reconstructing bone defects, and as a thin coating on metals like titanium and CoCrMo alloys for hip, knee, and dental prostheses [4]. Despite these excellent properties of HA, its rheological strength is far less than those required for bone tissue engineering materials [5]. Osteomyelitis, the implant associated bacterial infection, is the major problem worldwide responsible for the majority of implant failure cases. The number of implant associated infections is steadily increasing as increase in the number of implanted devices [6]. Since HA is used as a coating material of implants, however till now, only few reports were available to show the antimicrobial properties of HA-NPs and no reports on possible toxic mechanism on bacterial system. The most important application of bioactive ceramics such as hydroxyapatite has been the coating of orthopedic metal implants, at locations where a strong interface with bone is required. The introduction of an implant in the body is always associated with the risk of microbial infection. Infection is a major problem in orthopedics leading to implant failure. Implant-associated infections are the result of bacteria adhesion to an implant surface and subsequent biofilm formation at the implantation site. Implant infections are caused by both Gram + ve and Gram − ve bacterium. The antibacterial property of HA-NPs against Gram + ve bacteria *S. aureus* and *S. epidermidis* are analyzed before. But no complete work has been done in the area of antibacterial activity and mechanism against Gram − ve bacteria. In general the mechanisms behind the activity of NPs on bacteria are not yet fully elucidated. The three most common mechanism of toxicity proposed to date are uptake

* Corresponding author at: Department of Nanotechnology, SRM University, Kattankulathur 603 203, Kancheepuram District, Tamil Nadu, India.
E-mail address: gdevanandvenkatasubbu@gmail.com (G. Devanand Venkatasubbu).

http://dx.doi.org/10.1016/j.msec.2016.12.060
0928-4931/

of NPs followed by disruption of ATP production and DNA replication, generation of reactive oxygen species as a oxidative stress marker and direct damage to cell membranes [7]. Hence further studies were necessary to study the antibacterial activity and to explain the actual mechanism regarding the bacterial growth inhibition of HA-NPs.

In this current study, the HA-NPs were evaluated to be used as antimicrobial agent and an attempt was made to elucidate the toxic mechanism in microbial system and its cytotoxicity in mammalian cells.

2. Materials and methods

2.1. Synthesis of HA-NPs

HA-NPs were synthesized by wet chemical precipitation method involves the following reaction:

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)2 + 18H_2O$$

Aqueous suspension of calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (H_3PO_4, 85%), both of analytical grade, were used as reagents for the preparation. One litre of an aqueous suspension of H_3PO_4 (0.6 M) was slowly added drop by drop to one litre of an aqueous suspension of $Ca(OH)_2$ (1 M) with vigorous stirring for 2 h at room temperature [8]. Concentrated NaOH was added until a final pH of 11 was obtained. The white suspension obtained was washed thoroughly with deionized water and dried in an oven at 80 °C for 24 h. The dried powder was annealed at 500 °C for 3 h [9]. The phase purity of the synthesized HA-NPs was recorded using powder X-ray diffractometer (Seifert, JSO-DE BYEFLEX 2002, Germany). The average size and size distribution of the synthesized NPs were determined by Transmission Electron Microscopy (TEM). The sample was scanned at 200 kV (JEOL 2000Fx-II, Tokyo, Japan) high Resolution, analytical TEM with a W-source and a point-point resolution of 2 Å. The functional groups were analyzed by Fourier transform infrared spectroscopy using (Perkin Elmer Spectrum One Spectroscopy, Branford, CT, USA).

2.2. Antibacterial assay

The antibacterial activity of HA-NPs was tested against four implant associated pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Salmonella typhi* by micro-broth dilution assay, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) using Muller-Hinton broth (HiMedia, Mumbai, India). HA-NPs were serially diluted to obtain the final concentration ranges from 500 to 39 μg/ml in a sterile titer plate. 10 μl suspension of inoculum whose density was adjusted to that of 0.5 McFarland standard (approximately 1 to 2×10^8 CFU/ml) were added and incubated at 37 °C for 24 h. The MIC values were read at 630 nm in a ELISA Reader (Robonik Readwell plate ELISA Analyzer, India) and MIC_{50} values were calculated.

2.3. Growth curve profiling

To examine the bacterial growth curve in presence of HA-NPs, the test organisms were grown in Luria-Bertani (LB) media supplemented with MIC_{50} concentration of NPs. Subsequently, 2×108 CFU/ml of test organisms were added to the above broth as inoculum and all the flasks were put on shaker incubator (180 rpm) and incubated at 37 °C. The control group was maintained without NPs. The bacterial growth was indexed by measuring optical density at every 2 h (up to 20 h) at 600 nm using spectrophotometer (Shimadzu UV-Spectrophotometer UV-1601).

2.4. Determination of reactive oxygen species (ROS)

Generation of intracellular ROS was measured by the oxidation-sensitive fluorescent probe using DCFH-DA (Sigma-Aldrich). The bacterial cells were incubated for a period of 3 h with MIC_{50} concentration of HA-NPs. 1 ml of each culture was pelleted by centrifugation at 5000*g* for 5 min and resuspended in PBS containing 30 μg/ml DCFH-DA at 37 °C for 30 min in the dark. As positive control cells were treated with 10 μM H_2O_2 for 30 min added prior to the DCFH-DA and untreated cells were used as negative control. The cell suspension was transferred to 96-well plate and fluorescence was measured at an excitation wavelength of 480 nm and emission wavelength of 520 nm using a fluorescence multiwell plate reader (Perkin Elmer).

2.5. DNA fragmentation analysis

All the bacterial cultures were incubated for 24 h in Luria-Bertani broth supplemented with the MIC_{50} concentration of HA-NPs. DNA was extracted by resuspending the cell pellet in 200 μl of TEG buffer, 40 μl of 10% SDS and 5 μl of Proteinase K (20 mg/ml). The mixtures were then incubated at 56 °C for an hour. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) was added to the mixture and centrifuged for 10 min at 13,000*g* at 4 °C. The aqueous layer was digested with 800 μl of RNase (10 mg/ml) and DNA was precipitated from the aqueous layer by adding two volumes of absolute ethanol and 400 μl of 0.1 M sodium acetate. The resulting pellet was rinsed twice with 70% ethanol, air-dried, and dissolved in 1 ml of TE buffer. The DNA was then evaluated on a 0.8% agarose gel using ethidium bromide and the DNA pattern was documented by a gel documentation system (Bio-Rad).

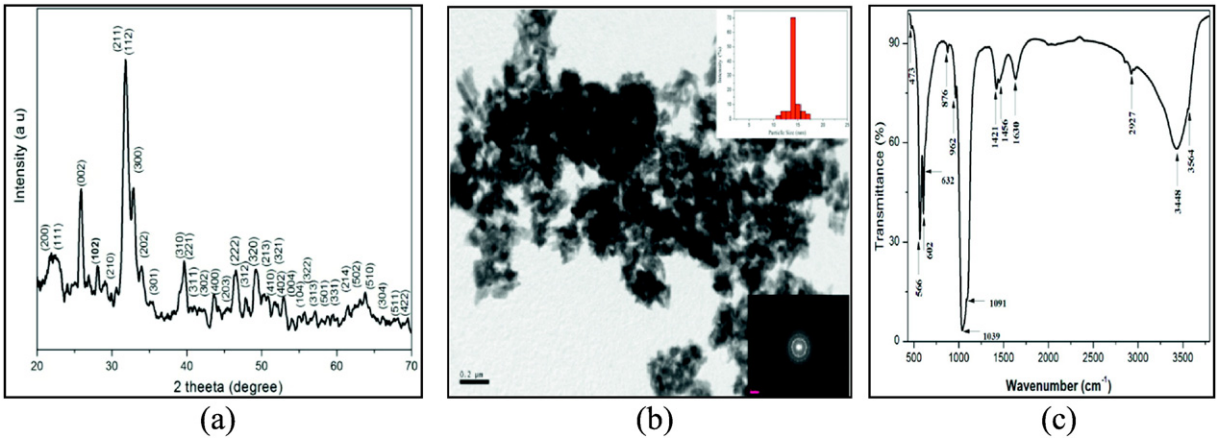

Fig. 1. a) XRD image of hydroxyapatite nanoparticles b) TEM image of hydroxyapatite nanoparticles c) FTIR image of hydroxyapatite nanoparticles.