Chapter 2

Experimental details

The second chapter presents the methodology for the development of 1- and 2-D HA nanocrystals. Various characterization techniques, such as XRD, FTIR, FESEM, EDX, and TEM, are performed to confirm the phase, morphology, atomic composition, and porosity of the samples. Surface chemistry, including surface charge, oxygen vacancy, and wettability, are also analyzed using zeta potential measurement, XPS, and contact angle measurement, respectively. Inductively coupled plasma-mass spectroscopy (ICP-MS) is also performed to understand the leaching response of different morphological HA nanocrystals. Cell culture, hemolysis, and antibacterial experiments are carried out to investigate the cytocompatibility, osteogenic activity, hemocompatibility, and antibacterial efficacy of different morphological HA nanocrystals. Further, related experiments for drug loading in HA nanocrystals and their release behavior to control the bacterial growth have also been performed.

2.1. Synthesis of different shapes of HA nanocrystals

The different morphologies of HA nanocrystals were synthesized using a facile HT method with the assistance of PVA as a capping agent. Initially, the solutions of 0.25 M $Ca(NO_3)_2.4H_2O$ and 0.15 M $Na_2HPO_4.2H_2O$ precursors were individually prepared in deionized (DI) water at room temperature. Afterwards, these solutions were successively added into 60 ml aqueous solution of 2.13 wt % of PVA under constant stirring at room temperature, which was followed by the adjustment of pH from 5 – 11 (depending on pH, the samples were named HA5, HA7.5, HA9, HA9.5, HA10, and HA11) with careful addition of NaOH solution at a temperature of 40ºC. Then, the milky white solution was transferred into a 500 ml Teflon autoclave and treated hydrothermally at a temperature of 200ºC for 12 h. Now, the resulting product was washed with DI water several times and dried at 90ºC in the oven for 24 h. Finally, the obtained powder was calcined at 700ºC for 2 h. A similar experiment was repeated again

at pH 9.5, except for the duration of HT treatment, which was performed only for 6 h. The samples, prepared at 6 and 12 h of HT time, were named HA-6h and HA-12h. Additional samples were also prepared by varying the amount of PVA from 2.13 to 6.13 wt % while keeping the other experimental parameters constant (pH 9.5, HT condition: 200ºC for 12 h). The samples, prepared at 2.13, 4.17 and 6.13 wt % of PVA, were denoted as HA-C1, HA-C2 and HA-C3, respectively.

2.2. Structural, physical, and chemical characterizations

To evaluate the phases of the prepared HA samples, the XRD measurements were done at room temperature using Rigaku Miniflex II Desktop X-ray Diffractometer with monochromatic Cu-Kα (λ = 1.5406 Å) radiation, step size of 0.02º, dwell time of 0.6 sec, and a scan rate of 2º/minute over the diffraction angle (2θ) of 10 – 80º. The peak profile fitting was performed using FullProf software to reveal the information about the changes in lattice parameters as per the morphology of HA nanocrystals. The infrared spectra were recorded in the region of 4000 – 400 cm^{-1} using a FTIR spectrometer (Bruker Tensor 27, Germany) to examine the functional groups, present in HA nanocrystals. Raman spectra was also recorded between 400 – 4000 cm^{-1} at 10 number of acquisitions, and 4 sec of acquisition time using a Raman spectrometer with He-Ne Laser (Power = 20 mW, λ = 532 nm) as an excitation source to examine the vibrational modes of molecular bonding, present in nHA samples.

The morphological and particle size analyses of HA samples were carried out using FESEM (Nova Nano SEM 450, FEI). The elemental composition, purity, and atomic ratio of Ca/P of prepared HA nanocrystals were determined by EDS, equipped with FESEM. The closer observation of morphology and porosity in HA nanocrystals was made with TEM (TECNAI 20G2, Thermo Fisher), operated at an accelerating voltage of 200 kV. Moreover, the high-resolution TEM images and selected area electron diffraction (SAED) patterns were also recorded for crystallographic analyses. TEM samples were prepared using the drop-casting

technique. The image analyses of HRTEM were carried out by Image J software. The further particle size and surface charge of nHA samples were examined using Zeta sizer ultra (Malvern Panalytical, ZSU5700).

The concentrations of oxygen vacancy on nHA samples were investigated using XPS (PHI 5000VersaProbeIII; XPS) with Al–Kα as the X-ray source. XPS survey spectra was collected at pass energy 280 eV. The data was analysed using Casa XPS software.

To study the ion leaching behavior of nHA powder in biological fluid, the concentrations of leached calcium and phosphorus ions in simulated body fluid (SBF) after a specified incubation period were examined using ICP-MS (Thermo Fisher Scientific). For this purpose, nHA powder was immersed in 10 ml SBF (pH: 7.4) and incubated for 3, 5 and 7 days at 37ºC. After the stipulated incubation period, the solution was diluted up to 5 times with DI water, and 5 % HNO_3 was subsequently added to digest the HA particles. The digested solution was then filtered with a 0.22 µm pore-sized syringe filter. The contact angle on HA nanorods, nanoplates, thin nanoplates, and control samples was measured towards DI water, PBS, and cell growth media using a contact angle measurement unit to examine the hydrophilicity of the sample surface.

2.3. Hemolysis assay

The hemocompatibility of different concentrations (0.2, 2 and 20 mg/ml) of HA nanorods, nanoplates, and thin nanoplates was evaluated using hemolysis assay**.** The assay was performed as per standard ASTM F 756-00. For this purpose**,** fresh rat blood was collected from animal laboratory of Department of Pharmaceutical Engineering, IIT (BHU), which was taken from retro-orbital puncture in sterile vacutainer tubes. The collected blood was centrifuged at 6000 RPM for 10 min at 4ºC to separate red blood cells (RBCs), and subsequent RBCs were rinsed several times with sterile saline. The washed RBCs were suspended in sterilized PBS (pH: 7.4) in the ratio of 1:10 (RBCs to PBS). 100 µl of each nHA dispersion was mixed with 200 µl

RBCs suspension. The remaining 700 µl PBS was further added to make the volume of the solution up to 1 ml, and incubated at room temperature for 12 h. 0.1 % Triton-X100 and PBS served as positive and negative controls, respectively. After incubation, these solutions were centrifuged at 4ºC and 6000 RPM for 10 min to remove the non-lysed RBCs. The collected supernatants were transferred into 96-well plate in triplicate, and the absorbance was taken in microplate reader at 540 nm to assess the concentration of lysed RBCs. The % hemolysis was calculated using the following formula,

$$\%\ hemolysis = \frac{OD_{sample} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}} \times 100\ \% \quad (2.1)$$

2.4. Cellular response

Both, quantitative and qualitative cell culture studies were performed to assess the *in vitro* cellular response of different morphological HA nanocrystals at varied concentrations, for which MG-63 osteoblast cell lines was procured from NCCS Pune, Maharashtra. Dulbecco's modified Eagle's medium (DMEM) was used as cell growth media after being supplemented with 10 % fetal bovine serum (FBS; Gibco) and 1 % antibiotic (antibiotic−antimycotic, Gibco). Initially, MG-63 cells (5th generation) were grown up to 60 – 70 % confluency in DMEM medium at 37ºC with 5 % CO_2 and 95 % relative humidity. Before seeding, autoclaved nHA powders were dispersed in sterilized PBS according to concentrations of 0.2, 2 and 20 mg/ml. To seed the samples, a certain amount of Trypsin – EDTA solution (0.25 %; Gibco) was added to the culture flask to detach the cells from the surface. Afterwards, 2×10^4 cells/ml were seeded on glass cover-slips (Gelatin-coated) in a 24-well plate and kept in an incubator for 12 h to adhere the cells. After fixed culture duration, 100 µl of each sample solution was exposed to the adhered cells along with the addition of 400 µl DMEM. The same amount of PBS was also added to a well with the adhered cells, which served as a control. The seeded samples were incubated for 3, 5 and 7 days at 37ºC with 5 % CO_2 and 95 % relative humidity.

2.4.1. Cell viability

The MTT [(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)] assay was performed to evaluate the viability of MG-63 cells on different concentrations of HA nanorods, nanoplates and thin nanoplates, and control after 3, 5 and 7 days of incubation. Following the specified incubation period, the media was removed from each well, and cultured cells were cleaned twice with PBS solution. Afterwards, 500 µl MTT solution (1:10; MTT: DMEM) was added into each well and incubated for 6 h for the formation of formazan crystals, which were then dissolved by dimethyl sulfoxide (DMSO) solution. The obtained purple-colored solution was placed into a 96-well plate, and their concentrations were evaluated using an ELISA microplate reader at 595 nm. The experiments were repeated three times (n =3). The outcome data were statistically analysed using SPSS software. To examine the significant difference among the different morphological samples, one-way analysis of variance (ANOVA) technique, along with the Tukey test at the significant value of $p \leq 0.05$ was used.

2.4.2. Fluorescence imaging

The adhesion and morphology of MG-63 cells were analyzed on different concentrations of HA nanorods, nanoplates, thin nanoplates, and control after 3 days of incubation. For this purpose, cells were seeded for 3 culture days. Thereafter, adhered cells on the sample surface, were fixed with 4 % paraformaldehyde for 30 min, which was then permeabilized with 0.1 % Triton-X100 for 10 min. After permealization, these cells were blocked by adding bovine serum albumin (BSA) at 4ºC for 1 h. Now, the cytoskeleton and nuclei of the cells were stained with Alexa Flour 488 (Invitrogen) and Hoechst (Invitrogen) dyes, respectively. The images of stained cells on the sample surface were captured using fluorescence microscopy (Nikon Eclipse LV 100 ND) at 10× magnification.

2.4.3. ALP (Alkaline phosphatase) evaluation

The ALP assay was conducted to investigate the effect of various shapes of HA nanocrystals at different concentrations on the osteogenic differentiation potential of MG-63 cells. ALP serves as an essential early marker of osteoblast differentiation, reflecting the capacity of cells to form new bone i.e., bone mineralization [1]. For this study, osteogenic media was prepared by mixing 0.2 mM ascorbic acid (Himedia) and 10 mM β-glycerophosphate (Himedia) in DMEM. After seeding the cells on samples in osteogenic media, ALP expression was evaluated after 7 and 14 culture days. Following fixed culturing days, the media was removed from each well and rinsed with PBS. Thereafter, the seeded cells were treated with 50 µl of 0.1 % Triton-X100 lysis solution for 10 min, which was subsequently supplemented with 200 µl of p-nitrophenyl phosphate (Himedia) solution (1 mg/ml). After incubation (37ºC for 1 h), the reaction was stopped by adding 2 N NaOH (Sigma) solution. The absorbance of the final solution was measured at 405 nm using a microplate reader.

2.5. Antibacterial Activity

The antibacterial activity of developed HA nanocrystals with different morphologies was investigated against pathogenic bacteria, gram-negative *E. coli* and gram-positive *S. aureus*. Both bacteria were procured in freeze-dried conditions from Microbial type culture collection (MTCC), Chandigarh, India. Quantitative experiment was done by MTT assay. In contrast, qualitative analysis was proceeded by live-dead assay and colony-counting test. A ROS assay was also performed to quantify the intracellular ROS production in bacterial culture, after treatment with HA nanocrystals. To conduct these experiments, the bacteria were initially cultured in the nutrient broth media at 37ºC for 12 h to obtain an exponentially grown bacterial culture. This healthy culture was subsequently diluted (0.1 optical density) to seed the HA samples of various concentrations, 0.2, 2 and 20 mg/ml. The glass coverslip was taken as a control.

The experiments were repeated three times (n = 3) and, in each experiment, triplicates of the samples were used. The outcome data were statistically analyzed using SPSS 20 software with a one-way analysis of variance (ANOVA) method, which is followed by the Tukey test at the significant value of $p \leq 0.05$ to compare the results among different morphologies of HA nanocrystals.

2.5.1. Quantitative assessment

2.5.1.1. MTT assay

The MTT assay was performed to evaluate the viability of *E. coli* and *S. aureus* bacteria after treatment with different concentrations of HA nanocrystals. 100 µl of autoclaved samples were seeded with 200 µl of bacterial culture (0.1 OD) in each well of the 24-well plate, and incubated at 37ºC for 8 – 10 h. After incubation, the media, containing HA nanocrystals, was removed from each well and washed with 1 X PBS twice. Following this, the reconstituted MTT (MTT: PBS; 1:10) was added to each well and further incubated at 37ºC for 2 h, which reacts with viable bacterial cells and forms the water-insoluble formazan crystals. Now, the MTT was replaced with DMSO to solubilize the formazan crystal, and the absorbance of the resultant solution was taken using an ELISA microplate reader at 595 nm. The antibacterial ratio of both the bacteria, treated with various concentrations of different morphological HA nanocrystals, was calculated as,

$$Antibacterial\ ratio\ (\%) = \frac{OD_B - OD_S}{OD_B} \times 100 \quad (2.2)$$

Where, OD_B and OD_S indicate the optical densities of blank and samples, respectively.

2.5.2. Qualitative assessment

2.5.2.1. Colony-counting test

The colony-counting test was used to examine the antibacterial potential of different morphological HA nanocrystals. Briefly, 100 µl of autoclaved samples were seeded with 200 µl of bacterial culture (0.1 OD) in each well of the 24-culture well plate, and incubated at 37ºC

for 8 – 10 h. After the specified incubation period, the treated bacterial culture was diluted to 10^5 times with sterile PBS. Now, 50 µl of subsequent culture was uniformly spread on agar plates using a L-rod and incubated at 37ºC for 12 h. After incubation, the bacterial colonies were formed on agar plates, which were counted using a digital colony counter. The antibacterial ratio of different concentrations of HA nanocrystals was calculated as,[2]

$$Antibacterial\ ratio\ (\%) = \frac{N_B - N_S}{N_B} \times 100 \quad (2.3)$$

Where N_B and N_S indicate a number of colonies, formed on blank and samples, respectively.

2.5.2.2. Live-dead assay

The live and dead bacterial cells were assessed using a fluorescence microscope (Nikon Eclipse LV 100 ND), after staining with SYTO 9 and Propidium iodide (PI) dyes for live and dead bacteria, respectively. In a 24-tissue culture well plate, 200 µl of bacterial culture (0.1 OD) was treated with 100 µl of samples on a glass coverslip, with an untreated coverslip as a control. After incubation at 37ºC for 8 – 10 h, the samples were washed twice with PBS and stained with 1 – 2 µl of SYTO 9 and PI mixture in equal proportions. Subsequently, they were further incubated in the dark for 15 min.

2.5.3. Reactive oxygen species (ROS) assay

ROS, such as $O_2^{\bullet-}$, H_2O_2 and hydroxyl radical ($^{\bullet}OH$), etc., are generally a byproduct of cellular oxidative metabolic process in mitochondria [3]. These ROS are typically neutralized by antioxidant enzymes, such as SOD and catalase, to protect bacterial cells. However, their elevated concentration destroys the neutralizing capacity of antioxidant defense, causing oxidative stress within the bacteria. This oxidative stress damages lipid membranes, proteins, and DNA, ultimately resulting in bacterial death [4-6]. It is well-known that nano-sized particles are susceptible to higher production of ROS due to their high surface-to-volume ratio, smaller size, and surface reactivity [7]. At the nanoscale, several factors influence the amount of ROS generation, including size, shape, surface charge, ion release, crystallinity, particle

agglomeration, and dissolution [8-10]. In this perspective, a number of ROS assays, including SOD, catalase, protein leakage, and LPO, were conducted for the quantification of $O_2^{\bullet-}$ radicals, H_2O_2, ROS-induced lipids and protein damage, respectively. Initially, 100 µl of each sample was seeded with 200 µl of bacterial culture (0.1 OD) for 8 – 10 h at 37ºC. After incubation, the bacterial culture was replaced with 1 mg/ml of lysozyme buffer and kept in an incubator for 2 h. Now, this buffer solution of each sample is centrifuged for 10 min at 10000 rpm and 4ºC, and the resultant supernatant is stored at 4ºC for further ROS experiments.

2.5.3.1. Superoxide dismutase (SOD) assay

SOD assay is a simple and flexible method for measuring SOD enzymatic activity, which was observed by the decrease in production of superoxide anions after treating *E. coli* and *S. aureus* bacteria with HA nanocrystals. For this assay, bacterial supernatant (200 µl) of each sample was mixed with SOD buffer, which was then followed by the successive addition of methionine (130 mM), ethylenediaminetetraacetic acid (0.5 mM), nitro blue tetrazolium (0.75 mM), and riboflavin (60 µM). The final reaction solution was exposed to fluorescence light for 6 min to turn into blue color, and the absorbance was taken at 560 nm. The absorbance is directly proportional to the number of superoxide anions in the reaction solution [11, 12].

2.5.3.2. Catalase assay

The catalase activity was determined according to the method, reported by Aebi [13]. It was measured by the dissociation rate of H_2O_2 free radicals into H_2O and O_2 after treating the bacterial cells with HA nanocrystals. For this experiment, the reaction solution was prepared by adding bacterial supernatant (100 µl) with 50 mM phosphate buffer (pH 7) and 30 mM H_2O_2. The optical density of the final solution was taken using a UV-visible spectrometer at 240 nm. The time-dependent decomposition of H_2O_2 was assessed by means of catalase activity/sec.

$$Catalase\ activity = \frac{2.3}{\Delta t} \times \log \frac{E_1}{E_2} \tag{2.4}$$

Where, E_1 and E_2 indicate the absorbance for H_2O_2 concentration at t = 0 and 30 sec, respectively.

2.5.3.3. Lowry protein assay

The ROS-induced protein damage of *E. coli* and *S. aureus* bacteria after treatment with HA nanocrystals was quantified according to Lowry protein assay with BSA as a standard [14, 15]. In this experiment, the bacterial supernatant (20 µl) of each sample was initially maintained at 1 ml with the addition of distilled water. Afterwards, the reaction solution was mixed with 5 ml solution of 2 % Na_2CO_3 (in 0.1 N of NaOH) and 0.5 % $CuSO_4$ (in 1.35 % potassium sodium tartrate) and held for 10 min. Following this, 500 µl of 50 % folin reagent was mixed and held under the dark environment for 30 min to turn the solution into blue color. The absorbance of the final reaction solution was measured at 750 nm.

2.5.3.4. Lipid peroxidation (LPO) assay

LPO assay was carried out to examine the ROS-induced lipid damage of *E. coli* and *S. aureus* bacterial cells after treatment with HA nanocrystals [16, 17]. For this experiment, bacterial supernatant (500 µl) of each sample was mixed with 500 µl of Tris HCl and incubated for 2 h. After specific incubation, 1 ml of trichloroacetic acid was added to the obtained solution and centrifuged for 10 min at 3000 rpm and 4 °C. The obtained supernatant was thoroughly mixed with an equal quantity of thiobarbituric acid and boiled for 10 min to change the color of the reaction solution to pink. Afterwards, 1 ml of distilled water was mixed, and absorbance was taken at 532 nm. The amount of malondialdehyde (MDA) per milligram protein was calculated as,

$$MDA/mg\ protein = \frac{OD\ (532) \times Reaction\ volume \times 10^9}{Sample\ volume \times 1000 \times Extinsion\ coefficient\ of\ MDA} \quad (2.5)$$

Where the value of the extinsion coefficient for MDA is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.6. Drug delivery

2.6.1. Drug loading and release experiments

The *in vitro* investigation of drug loading and release response from HA nanocrystals was started with the development of a standard calibration curve of DOX, plotted by taking the absorbance of DOX solutions (5 to 50 μg/mL) at 350 nm using a UV-visible spectrophotometer. To prepare DOX-loaded HA nanocrystals, the solution technique was used. This process involved dissolving the HA nanocrystals and 10 wt % DOX in DMSO with continuous overnight stirring to ensure complete adsorption of the drug. The obtained mixture was dried in vacuum oven at 45°C. The resulting powder was stored under ambient conditions. The drug loading efficiency of the HA nanocrystals was determined using a specific formula [18],

$$Drug\ loading\ efficiency = \frac{Conc.\ of\ inital\ drug - Conc.\ of\ free\ drug}{Conc.\ of\ inital\ drug} \times 100 \qquad (2.6)$$

The drug release experiments were carried out in a PBS buffer with a pH of approximately 7.4. For this, 2 mg of DOX-HA nanocrystals was placed inside a dialysis bag and immersed in 40 mL of the release medium, which was then kept in an incubator shaker, set at 200 rpm and 37°C. At designated time intervals, aliquots were taken from the release medium, with an equal volume of fresh PBS, added to maintain similar conditions. The DOX concentration in the collected samples was measured by taking the absorbance at 350 nm.

2.6.2. Phase evaluation, morphological, and chemical characterizations

The DOX loading on the surface of HA nanocrystals was confirmed using FTIR spectrometer (Bruker Tensor 27, Germany) and XRD (X-ray diffractometer; Rigaku Miniflex II) techniques. FTIR spectra was recorded across the wavenumber ranging from 4000 – 400 cm^{-1} to reveal the functional groups, associated with HA nanocrystals before and after loading of DOX drug. XRD patterns were scanned at 2º/min using Cu-Kα (λ = 1.54 Å) monochromatic beam with diffraction angle of 10 – 80º. The morphological changes in HA nanocrystals after loading of

DOX was observed using FESEM (Nova Nano SEM 450, FEI) and TEM (TECNAI 20G2, Thermofisher). The nature of molecular interaction between HA and DOX was examined using XPS (Thermo Fisher Scientific) analyses. The surface area of developed nHA samples was examined by nitrogen physisorption at 77 K using BET analyser (BELLSORP MAX II & BELCAT-II; MicrotracBEL Corp).

2.6.3. Cellular response

The cytocompatibility of DOX-loaded HA nanocrystals was assessed both, quantitatively and qualitatively. For this purpose, MG-63 cells were grown up to 60 –70 % confluency in Dulbecco's modified Eagle's medium (DMEM; supplemented with 10 % fetal bovine serum and 1% antibiotic) at 37°C in 5% CO_2 incubator. DOX-HA nanocrystals were dissolved in PBS at increasing concentrations of 10, 100, and 200 µg/ml. The 2×10^4 cells/ml were seeded on each sample using the same procedure, as discussed in section 2.4. The MTT assay was conducted to assess the MG-63 cell viability on DOX-HA nanorods (DOX-HNRs), DOX-HA nanoplates (DOX-HNPTs), DOX-HA thin nanoplates (DOX-HTNPTs), and control after 24, 48, and 72 h of incubation period. The same protocol was used to perform the experiment as discussed in section 2.4.1. For fluorescence imaging of MG-63 cells cultured on DOX-HNRs, DOX-HNPTs, DOX-HTNPTs for 3 days, the adhered cells on glass cover slip were fixed as per standard protocol. The cells were then successively stained with Alexa Fluor 488 and Hoechst dyes, and images were taken under fluorescence microscopy (Nikon Eclipse LV 100 ND).

2.6.4. Hemolysis assay

The hemocompatibility of DOX-HA nanocrystals was also evaluated using hemolysis assay. The assay was performed as per standard ASTM F 756-00, which is already discussed in section 2.3.

2.6.5. Antibacterial response

Both, quantitative (MTT assay) and qualitative (Live-dead and colony-counting) experiments were conducted to assess the antibacterial response of DOX-HNRs, DOX-HNPTs, and DOX-HTNPTs at increasing concentrations (10, 100, and 200 µg/ml) against *S. aureus* bacteria. The protocols were already mentioned in section 2.5.1. and 2.5.2.

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