1- and 2-Dimensional (D) Hydroxyapatite Nanocrystals: Synthesis, Growth Mechanism, and Their Effects on Osteogenesis, Antibacterial Activity, and Drug delivery

Thesis submitted in partial fulfillment for the

Award of Degree

Doctor of Philosophy

by

Urvashi Kesarwani

DEPARTMENT OF CERAMIC ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY
(BANARAS HINDU UNIVERSITY)
VARANASI – 221005
INDIA

Roll No. 18031505 **2025**

Chapter 7

Conclusions and future perspectives

The present chapter briefs the major outcomes of the studies, performed as part of the thesis, such as development and characterizations, including cellular and antibacterial response of 1- and 2-D HA nanocrystals, along with drug loading and their release response to control the bacterial growth.

Conclusions

1. 1- and 2-D HA nanocrystals were successfully synthesized using highly biocompatible and low-cost PVA as a surface capping agent via HT method, for which various processing parameters, such as pH of the solution, HT time, and PVA concentrations, were systematically varied to achieve the desired morphology. The HA nanorods (70 – 500 × 22 – 62) nm^2 and nanoplates (200 – 500 × 60 – 250 × 7 – 66) nm^3 were formed at pH 5 and 9.5, respectively, under the HT condition of 200ºC for 12 h and PVA concentration of 2.13 wt %. The thin HA nanoplates (Thickness: 7 – 23 nm, length: nm to μm) were produced at a pH of 9.5, PVA concentration of 2.13 wt %, and HT temperature and time of 200ºC and 6 h, respectively. Using HRTEM images, the preferential crystal growth direction was identified along c- and a-axes for HA nanorods, nanoplates and thin nanoplates, respectively. The possible molecular mechanism, governing the formation of different morphological HA nanocrystals was also successfully discussed in detail and verified it based on previous studies.
2. XPS, zeta potential, and contact angle measurements suggested that the surface oxygen vacancy, surface charge, and hydrophilicity are the highest in HA thin nanoplates, moderate in HA nanoplates and lowest in HA nanorods. Particularly, the zeta potential value was observed to be – 6.1, – 44.2, and – 48.1 mV for HA nanorods, nanoplates and thin nanoplates, respectively.

3. The ICP-MS experiment revealed that the leaching of Ca^{2+} and P^{5+} ions from HA thin nanoplates is comparatively higher than HA nanoplates. The ion leaching profile of HA nanorods is observed to lie between HA thin nanoplates and nanoplates. Such leaching behavior of HA nanorods, nanoplates, and thin nanoplates is directly related to their crystallinity, which was calculated to be ~ 89, 97 and 86 %, respectively. Higher crystalline nHA suggests lower dissolution ability.
4. *In vitro* cell culture studies revealed that negatively-charged HA nanoplates significantly accelerate the cell adhesion, proliferation and differentiation as compared to that of less negatively charged HA nanorods. However, despite of higher surface charge and better wettability of HA thin nanoplates as compared to HA nanoplates and nanorods, it revealed the lowest cytocompatibility and osteogenic response, which is attributed to the leaching of relatively higher concentration of Ca^{2+} ions (3.163 ppm) that influx within the cells and enhances the intracellular calcium ions which disturbs the metabolic process, ultimately leading to cell death.
5. The hemolytic study demonstrated that the toxicity of HA nanorods, nanoplates, and thin nanoplates is less than 5 %, which is considerably compatible with RBCs at various nHA concentrations (0.2, 2 and 20 mg/ml), indicating good hemocompatibility.
6. Quantitative and qualitative antibacterial experiments of various concentrations (0.2, 2 and 20 mg/ml) and morphologies of HA nanocrystals revealed that HA nanorods and thin nanoplates exhibited good antibacterial response at concentrations of 0.2 and 20 mg/ml, respectively. Even 20 mg/ml concentrated HA thin nanoplates showed the highest antibacterial response among all the tested concentrations and other samples. Moreover, the least antibacterial response was observed for HA nanorods at the concentration of 2 mg/ml. The mechanism for the bactericidal effect of HA nanocrystals was also revealed by SOD, catalase, protein estimation, and LPO assays. The bactericidal effect of 0.2 mg/ml

concentrated HA nanorods was due to over production of ROS within bacterial cells. In contrast, 20 mg/ml concentrated thin HA nanoplates showed antibacterial potential due to the synergistic effect of ROS generation and their electrostatic repulsion with bacteria.

7. Antibiotic DOX drug was successfully loaded on 1- and 2-D HA nanocrystals using the solution technique. The DOX loading efficiencies of HA nanorods, nanoplates, and thin nanoplates were evaluated to be 45.5, 62.2, and 73.3 %, respectively. The successful DOX loading was confirmed through FTIR and XRD, while morphological changes before and after DOX loading was also revealed by FESEM and TEM. Among the tested samples, HA thin nanoplates showed the most prolonged DOX release profile, releasing 49 % of DOX within 48 h, followed by HA nanoplates and HA nanorods, which showed release profiles of 59.5 and 69 % in 48 h, respectively.
8. The differential DOX loading and release rate as per shape of HA nanocrystals was thoroughly explained on the basis of surface area of HA and their nature of interaction with DOX. This interaction behavior of DOX as per different nHA morphologies is related to their surface area, which was calculated to be 18, 25.5 and 34 m^2/g for HA nanorods, nanoplates, and thin nanoplates, respectively. Due to lower surface area, HA nanorods possess fewer number of reactive sites than HA nanoplates and thin nanoplates, allowing major amount of drug loading through H-bonding interaction (lower energy is required to form). On moving from HA nanorods to nanoplates, number of reactive sites increases. It facilitates DOX loading through both, primary and secondary bonding.
9. *In vitro* cell culture studies demonstrated that DOX-HA nanocrystals formulations effectively promoted cell adhesion and proliferation, with DOX-HNRs, DOX-HNPTs, and DOX-HTNPTs exhibiting cell viability in decreasing order. The relatively lower viability observed for DOX-HTNPTs was attributed to their highest DOX loading efficiency and sustained drug release profile. Additionally, hemolysis studies also confirmed that all

DOX-HA nanocrystals possess good hemocompatibility (< 5% RBC lysis). Furthermore, *in vitro* antibacterial studies revealed that, unlike HA nanorods and free DOX, the prolonged DOX release from HA thin nanoplates leads to nearly complete bacterial inhibition at a concentration of 200 μg/ml.

Overall, among all the developed 1- and 2-D HA nanocrystals, HA nanoplates show superior cell adhesion, proliferation, osteogenic differentiation, and no appriceable toxicity towards RBCs as compared to those of HA nanorods and thin HA nanoplates. This indicates that HA nanoplates exhibit good biocompatibility, hemocompatibility, and osteogenic activity. Such bone-mimicking morphology of HA nanoplates can be a potential breakthrough biomaterial for bone tissue engineering applications. Moreover, HA thin nanoplates showed the highest antibacterial response at elevated concentration as compared to other samples. It can, therefore, be suggested that the antibacterial activity of HA nanocrystals is morphology and dose-dependent. Among the tested HA morphologies, HA thin nanoplates exhibited the highest DOX loading and the most sustained DOX release profile, followed by HA nanoplates and nanorods. Antibacterial studies revealed that, unlike free DOX, the prolonged DOX release from HA thin nanoplates leads to nearly complete bacterial inhibition at 200 μg/ml, highlighting their potential as an effective antibiotic vehicle for bacterial eradication.

Future perspectives

1. The present study developed HA nanoplates with dimensions of 200 – 500 nm in length, 60 – 250 nm in width, and 7 – 66 nm in thickness. Some of the HA nanoplates precisely match the dimensions of natural HA. Therefore, experimental parameters should be further optimize to achieve all the HA nanoplates with dimensions precisely matched to the dimensions of natural HA.
2. Since the 3-D hierarchical structure of living bone is primarily composed of 2-D HA nanoplates, impregnated within a collagen fibril matrix. Therefore, the biological scaffold

based on HA nanoplates and its composites with collagen should be developed, which may be a potential alternative to the natural bone scaffold.

3. A bio-printer can be utilized to create a cell-laden HA nanoplates/collagen scaffold to replicate the microenvironment of living bone for cells.
4. Owing to bioelectric nature (e.g., piezoelectricity), natural living bone induces a microelectric field which promotes the metabolic activities of the bone, such as cell proliferation, differentiation, bone remodeling, as well as fracture healing. Therefore, piezoelectric biomaterials have been suggested as a potential prosthetic implant for bone tissue engineering application. HA shows piezoelectricity due to the presence of OH^- groups, and collagen is natural piezoelectric biopolymer. In this regard, the future studies could be based on piezoelectric measurement of HA nanocrystals and their composite with collagen. It is expected that various morphologies of HA nanocrystals can show different piezoelectric strain coefficients. Moreover, HA nanoplates/collagen scaffold could mimic the piezoelectric response of living bone.
5. The measurement of hardness, compressive strength, and fracture toughness can be performed to investigate the effect of different morphological HA nanocrystals.

List of Publications and Patent

1. **Urvashi Kesarwani**, Bikramjit Basu, and Ashutosh Kumar Dubey, "1- and 2-dimensional (1 D/2 D) hydroxyapatite nanocrystals: A deep insight into synthesis strategies and multidimensional applications", Applied Materials Today, 36 (2024) 102062.
2. **Urvashi Kesarwani** and Ashutosh Kumar Dubey, "Antibacterial efficacy of bone mimicking-hydroxyapatite nanoplates with varying morphology", Inorganic Chemistry Communications, 174 (2025) 113918.
3. **Urvashi Kesarwani** and Ashutosh Kumar Dubey, "Cytocompatibility and osteogenic response of 1- and 2-dimensional (D) nanostructured hydroxyapatite: Influence of surface chemistry, ion release and hydrophilicity", Inorganic Chemistry Communications, 173 (2025) 113824.
4. Ashutosh Kumar Dubey and **Urvashi Kesarwani**, "A Method of Preparation of 2D Bioceramic Nanoplates", Patent Application No.: 202311068646, 4-12-2024 (Indian Patent).
5. **Urvashi Kesarwani,** Subham Sekhar Mandal, Pralay Maiti, and Ashutosh Kumar Dubey, "Natural bone-mimicking hydroxyapatite nanoplates as a novel antibiotic drug vehicle: A comparative study with nanorods", Journal of Drug Delivery Science and Technology 114 (2025) 107474.
6. **Urvashi Kesarwani,** Ashutosh Kumar Dubey, Asish Kumar Panda, B Basu, Jonny, and J Blaker, "Bioelectronic medicine", Current Science, 122 (2022) 373.
7. **Urvashi Kesarwani** and Ashutosh Kumar Dubey. "Cytocompatibility, Osteogenic activity, hemocompatibility of different-sized hydroxyapatite nanorods" (Under preparation).

8. Kuntal Kumar Das, **Urvashi Kesarwani**, Ravi Prakash, Pralay Maiti, Om Shankar, and Ashutosh Kumar Dubey, "Piezoelectric catalyst $BaTiO_3$ and $K_{0.5}Na_{0.5}NbO_3$ induced cellular and antibacterial response in poly (vinylidene fluoride) for self-powered implants for orthopedic applications", Catalysis Today 452 (2025) 115255.