CHAPTER 5
NANO-HYDROXYAPATITE/β-CD/CHITOSAN NANOCOMPOSITE FOR POTENTIAL APPLICATIONS IN BONE TISSUE ENGINEERING
5.1 Introduction

Bone is a highly vascular, living and dynamic tissue wondrous for its union of regenerative ability and mechanical properties and hence a masterpiece from nature. It is a highly organized tissue which assembles from nano- to macroscales to build complex structural interconnected porous network. Bone repair or regeneration is a prevalent and complex clinical issue in orthopedic surgery. Over 2.2 million bone transplantation procedures are performed annually worldwide in different fields including dentistry, orthopedics and neurosurgery. There is a continued history of employing autografts and allografts in the treatment of bone deformities. Although autografts plays better role in terms of biocompatibility and other aspects, they require second surgery to procure donor bone from the patient’s own body. On the other hand, allografts are the attractive option because of their high availability. Nevertheless the use of allografts are inherently limited by risk of infections and immune responses, which may even intricate other health problems affecting quality of life. Therefore, to circumvent problems linked with existing treatments, the design and development of biomaterial scaffolds to promote bone regeneration has been a thrust area demanding synthetic bone tissue engineering substitutes (e.g., ceramics, polymers, metals, and organic bone substitutes) [1]. The requirements for an ideal biomaterial bone graft that would be of significant importance to the clinicians includes adequate mechanical properties, biocompatibility, controlled bioresorbability, non-toxicity, inexpensive nature and bioactivity. These properties help in the formation of a bond between the host tissue and the implant material eventually supporting the bone regeneration process [2]. The nanoscale materials and composites with diverse chemical constitutions provide optimal features such as size and chemistry for cell interaction and bone regeneration. The nanomaterials enhance protein adsorption, cell adhesion, proliferation, and differentiation compared to conventional materials [3]. In continuation to our earlier successful attempts in the synthesis of nanocomposites [4-6] for bone tissue engineering applications [7-8], we extended our research towards the synthesis of polymeric nanocomposites incorporating starting materials of natural origin having precise control of properties of appropriate architecture, mechanical properties, and degradation rate at different reaction temperatures. Keeping in view the fact that inorganic–organic composite materials are progressively important because of their exceptional features arising out of synergism between the properties
of the components involved, the polymeric nanocomposites have been synthesized in order to develop materials with enhanced mechanical features and bioactivity by unanimous integration of properties of the various components [5]. A series of naturally derived polymers such as gelatin, collagen, chitosan, starch [8] and chemosynthetic polymers such as polycaprolactone (PCL), polyethylene glycol, poly (lactic-co-glycolic acid), and polyurethane have been broadly employed in the biomedical and pharmaceutical field [9,7]. Among these polymers, chitosan being the only cationic polyheterosaccharide in nature composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues, has earned marked interest in orthopedic and other biomedical applications because of its excellent physico-chemical properties such as low-toxicity, biocompatibility, biodegradability, mucoadhesive nature etc. [10]. Many studies have been executed on the diverse chitosan-based implants concluding that chitosan could affect all stages of wound repair in experimental animal models [11]. The biological and mechanical properties of chitosan for bone replacement can be improved by combining it with calcium phosphates. A number of researches have captivated interests on key bone mineral and the most ubiquitous calcium phosphate, hydroxyapatite (HA) \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\) and its composites for bone repair materials due to its good bone-bonding properties and osteoconductivity which have been used widely in dental and orthopedic surgery to fill bone defects [12]. Many attempts have been made to synthesize binary systems with hydroxyapatite adding polymers such as chitosan, gelatin, alginate etc. resulting a promising material for bone grafts, but nowadays ternary systems with superior properties are receiving immense attention and favor in comparison to chitosan and chitosan/HA scaffolds for bone tissue engineering by introducing component like collagen, carbon nanotube, poly (caprolactone), carboxymethyl cellulose, montmorillonite, etc. [13,14].

Cyclodextrins (CDs), the water-soluble cyclic oligosaccharides, composed of \(\alpha\)-D-glucopyranoside units linked in 1–4 manners, are synthetic substances obtained from the enzymatic degradation of starch, which is one of the most fundamentally natural and renewable polysaccharide. CDs belong to the group of cage molecules having the core of their structure composed of a dimensionally stable hydrophobic cavity that can trap or encapsulate other molecules leading to a “host–guest” type relationship that can improve the chemical, physical and biological properties of the guest molecule.
and hence finding applications in the fields of pharmacy, food, chemistry, chromatography, catalysis, biotechnology, agriculture, cosmetics, hygiene, medicine, textiles, and the environment. The naturally occurring CDs are α, β and γ CDs having 6, 7 and 8 glucopyranose units, respectively, among which β-Cyclodextrin (β-CD), also known as cycloheptaglucan, being the most accessible, the lowest-priced and generally the most functional having the same chemical structural unit (glucose molecules) as cellulose [15]. Since CDs are considered as pharmaceutical excipients for numerous drug formulations which have been validated by US FDA, recently, an alendronate-β-cyclodextrin (ALN-β-CD) conjugate for local delivery of therapeutic agents to the bone and teeth has been synthesized and further studied as a delivery system for prostaglandin E1 for the treatment of bone defects which concluded that the β-CD derivative showed a robust bone anabolic effect and favored the process of bone regeneration [16]. It has also been reported in a recent investigation, that hydroxyapatite (HA) have been functionalized with cyclodextrin polymers resulted into a system capable of loading and progressively releasing lipophilic antibiotics, overcoming the infection caused by HA calcium phosphate bioceramics adopted for bone regeneration applications and orthopedic surgery, to fill bone defects [17]. Therefore, it is reasonable to design a nanocomposite system to exploit the combination of inorganic and organic phases that not only imparts bone with unique mechanical properties and a reservoir for minerals such as calcium and phosphate but also serves as a medium for diffusion and release of biological substances [18]. As a part of our curiosity to enhance the biomedical properties of chitosan/HA scaffold, it was thought worth focusing to synthesize n-HA/β-CD/CS nanocomposite at three different reaction temperatures by incorporating β-cyclodextrin in nano-hydroxyapatite/chitosan nanocomposite to form a ternary system through co-precipitation approach. To the best of our knowledge, no scientific study about the standalone application of cycloheptaglucan/β-CD based tri-component nanocomposite scaffolds with chitosan and nano-hydroxyapatite (n-HA) for bone regeneration applications have been explored till date. A series of detailed experiments were conducted to characterize and to evaluate various physico-chemical studies along with several essential in-vitro studies to monitor biological performances of these nanocomposites. In addition to above characterization, the biocompatibility was investigated in direct contact with human osteoblasts MG-63 cell line through in vitro analysis.
5.2 Experimental

5.2.1 Materials and Methods

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), Chitosan (CS) with degree of deacetylation (>85%), Phosphate Buffer Saline (PBS), and Dulbecco’s modified Eagle’s medium (DMEM), Triton lysate were purchased from Sigma-Aldrich (USA) and Invitrogen, USA, respectively. β-Cyclodextrin (β-CD), [Ca(NO₃)₂·4H₂O] (99%), (NH₄)₂HPO₄ (DAHP) (99%), NaOH (>97%), CH₃COOH (99.8%), DMSO, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, NaCl, CaCl₂, Na₂SO₄, tri-(hydroxylmethyl) aminomethane [TRIS], sodium dodecyl sulfate, p-nitrophenyl phosphate, Diethanolamine, Glutaraldehyde, EDTA, Ethanol, HCl and ammonia solution (25%) have been procured from Merck, Mumbai, India. All chemicals were of reagent grade and were used as received, and deionized water was used in all solutions and reagents throughout the experiment.

5.2.2 Preparation of n-HA

HA nanoparticles (n-HA) were prepared via wet chemical approach by the reaction between Ca(NO₃)₂·4H₂O and (NH₄)₂HPO₄ (DAHP) at a stoichiometric Ca/P ratio of 1.67. The pH value during the reaction being maintained above 10 by the addition of ammonia (25%) solution. The resulting precipitate so obtained was rinsed with water until the filtrate became neutral and then dried at 80°C [19,8].

5.2.3 Preparation route of n-HA/β-CD/CS nanocomposites at different temperatures.

Figure 1 schematically illustrates the synthetic pathway of the n-HA/β-CD/CS-(RT,HT,LT) nanocomposites via simple co-precipitation approach by mixing three aqueous solutions prepared as follows:

CS powder (1g) dissolved in 2 wt% aqueous acetic acid solution which was made up into 100 ml DI water followed by stirring for 24h to achieve a homogeneous suspension (solution I). The synthesized HA nanoparticles (1g) was dispersed in 100 ml DI water by sonication for 30 min (solution II). The β-cyclodextrin powder (1g) was dispersed in 100 ml lukewarm DI water by sonication for 30 min (solution III). The reaction proceeded by mixing the solutions I and II drop wise until the contents were thoroughly mixed followed by drop-wise addition of solution III. The reaction
mixture thus obtained was divided into three equal portions and was kept under the three temperatures for 48 hours as given below:

(i) at room temperature (n-HA/β-CD/CS-RT) under constant stirring at 1200 rpm maintaining pH in the range of 10-11 by using 0.5 M NaOH solution in order to accelerate the nucleation of n-HA expected at high pH value followed by stirring for 24 h at 1200 rpm. (ii) the reaction mixture frozen at -20 °C (n-HA/β-CD/CS-LT) while maintaining pH in the range of 10-11, (iii) at high temperature (n-HA/β-CD/CS-HT) at 80 °C under constant stirring at 1200 rpm maintaining pH in the range of 10-11. The products thus obtained were aged for another 24 hours which were filtered and washed several times with deionized water until the filtrate became neutral and dried in vacuum at 60 °C. In parallel, the CS/n-HA nanocomposite was also prepared via co-precipitation method in order to compare the various properties with that of the proposed nanocomposites.

Figure 1: Schematic illustration of the synthetic pathway of n-HA/β-CD/CS-(RT,HT,LT) nanocomposites.
5.3 Characterizations

5.3.1 Physicochemical and in-vitro screening

The dried products were analyzed by the different techniques given as follows. The size of the particles was examined by transmission electron microscopy, (TEM, Hitachi H-7500 Japan) 120 kV. SEM images at different magnifications were taken using Scanning electron microscope JEOL-JAPAN, equipped with an energy dispersive X-Ray spectroscopy EDX for bulk composition and elemental analysis. Atomic force microscopy (AFM, XE-100, park systems, Korea) in tapping mode was used to investigate topography and roughness of the synthesized nanocomposites. The samples for AFM imaging were made by drop casting a diluted suspension of nanocomposites onto a cleaned silicon substrate and drying at 50 °C. Topography of the surface was captured in tapping mode imaging with 10 x 10 μm² scan size and the root mean square (RMS) roughness was calculated from processed images. The FTIR spectra of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites scaffolds were recorded on (FTIR; Interspec 2020, spectrolab U.K) in KBr in frequency range of 4000-400 cm⁻¹. The crystallinity and phase of the samples was studied by X-ray diffraction (XRD) data measured on Philips PW1710 diffractometer with Cu Kα radiation at 1.540 Å in the range of 10⁰- 80⁰ at 40 kV. The thermal stability of the samples was evaluated by thermogravimetric analysis (TGA) and differential thermal analysis (DTA) studies of the samples carried out on Shimadzu system (DTG-60H Japan). The samples were heated from 30 °C to 800 °C at the rate of 10 °C/min in the nitrogen atmosphere. The shore hardness measurements of the samples were performed on shore hardness instrument prepared by Coats Machine Tool Co. Ltd, London. The compressive strength of the prepared nanocomposites was measured using a universal mechanical testing machine (INSTRON 5967, USA). The testing conditions were at room temperature. The crosshead speed was set at 1 mm/min and the load was applied until the samples were fractured. Three parallel samples were tested for each nanocomposite sample, and the mean value of the compressive strength, compressive modulus and shore hardness of different nanocomposites were given.
5.3.2 Statistical analysis

All quantitative data are expressed as mean ± standard deviations with n = 3. Statistical analysis was carried out using student’s t-test. A value of p < 0.05 was considered to be statistically significant [20].

5.3.3 Immersion in Simulated Body Fluid (SBF) Study: in–vitro mineralization test

The in-vitro bioactivity of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites was investigated by incubating the pellets (6mm in diameter and 2mm in thickness) made from dried samples of nanocomposites immersed in a beaker containing 20 ml of SBF solution [7,8] (having ion concentrations nearly equal to that of human blood plasma) oscillating at 37.0 ± 0.5 °C in a thermostatic bath to allow the soaking of SBF solution [21,22]. All operations were conducted in a laminar airflow hood to avoid bacterial infection. The pellets were withdrawn from SBF after soaking for the designated period i.e., 2 and 4 weeks and gently rinsed with DI water and dried. The apatite-forming capability of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite scaffolds was assessed through SEM and EDX.

5.3.4 Swelling Ratio assessment

Equal amounts of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposite samples were taken and their dry weights were noted as \( W_d \) [23]. The nanocomposite samples were then immersed in 20 ml SBF solution [pH 7.4] for the designated time period [1, 10, 20 and 30 days] at 37 °C. The samples were removed and blotted on filter paper to clear absorbed water and wet weights of the nanocomposite scaffolds were noted as \( W_w \). The experiment was performed in triplicates. The ratio of the swelling was determined using the Equation 1:

\[
SR = (W_w - W_d) / W_d
\]  

Where SR is the swelling ratio.

5.3.5 In-vitro degradation study

Degradation is another decisive property of a nanocomposite to be investigated in bone tissue engineering. In the case of an ideal biodegradable material, as the proliferation of bone into the scaffold promulgates and the bone cells build an
infrastructure naturally, the initial supporting scaffold is degraded and passes the mechanical stress and strain to the neo-tissue structure [24]. All the nanocomposite samples were equally weighed and the dry weights were noted as $W_o$. They were then immersed 10 ml of PBS containing lysozyme at a concentration analogous to that of circulating levels of blood [10,000 U/l]. Incubation was carried out at different time intervals (7, 14, 21 and 28 days) at 37 °C [25]. The samples were washed with DI water to remove any ions adsorbed on the surfaces and blot dried using filter paper at the completion of incubation period. Then wet weights were noted as $W_t$. The experiment was carried out in triplicates. The degradation percentage of the nanocomposite scaffolds was calculated by the following formula:

$$\text{Biodegradation (\%)} = \frac{W_o - W_t}{W_o} \times 100$$  \hspace{1cm} (2)

### 5.3.6 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay:

**In-Vitro Cell survivability assay:**

In order to evaluate the cell-nanocomposite interactions preliminary in-vitro experiments were performed to determine the cytotoxicity of the nanocomposites. A detailed experimental design includes following:

For investigating cellular toxicity of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide)] assay was carried out by culturing human osteoblasts like MG-63 cells (NCCS, Pune, India). In brief, MG-63 cells at a density of $0.5 \times 10^4$/well were seeded onto CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites at various concentrations (0-128 µg/ml) in 96-well culture plates [26]. The plates were incubated at 37 °C and 5% CO₂ for 24 h. After 24 h of incubation, the samples were removed from the respective wells and the wells were washed with phosphate-buffered saline (PBS, pH = 7.4). Only those cells that are adherent to the well walls were found viable and incubated with 0.5% MTT solution. The viable cells reduce the MTT into insoluble formazan precipitate by mitochondrial succinic dehydrogenase. After 4 h of incubation, the media containing MTT was gently removed and 0.1% dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. After these procedures, the absorbance of the content of each well was determined at 570 nm with a UV spectrophotometer. Cell viability (percent) was calculated and plot was generated using excel (Microsoft office-2007).
5.3.7 Antibacterial assessments

The antimicrobial properties of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites were evaluated by means of the microdilution method using 96 microwell plates, according to Clinical and Laboratory Standards Institute procedures (NCCLS) [27] against the following reference bacterial strains: Staphylococcus aureus (ATCC 25923), Listeria monocytogens (MTCC 657), Escherichia coli (ATCC25922), Vibrio cholera (MTCC 3906). Liquid culture for test microorganisms was grown overnight in Tryptone Soya Broth (Oxoid, UK) for bacterial growth at 37 °C before testing. The cultures were then centrifuged at 224 × g for 25 min to apportioned cells from culture broth and suspended at pH 7.3 in phosphate buffer saline (PBS). The dilution of suspensions was performed to adjust the number of cells to 1 × 10^7–1 × 10^8 CFU/ml. All experiments were performed in triplicate. The minimum inhibitory concentration (MIC) was the lowest concentration of the nanocomposite samples that caused 100% inhibition of microbial growth while Minimum bactericidal concentration (MBC) was the lowest concentration resulting in >99.9% reduction of the initial inoculum. The incubation time and temperature depended on the microbial species. The starting inoculum was 1.0 × 10^7 CFU/ml. Varied concentrations of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites were tested.

5.3.8 Hemocompatibility assessment

5.3.8.1 Protein adsorption

To determine the protein adsorption performance, the CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposite scaffolds were pre-washed with PBS, air dried and incubated with 500 μL of fetal bovine serum (FBS-GIBCO) at 37 °C for 2 h. The nanocomposite scaffolds were rinsed gently with PBS to eradicate unattached proteins after incubation. For the removal of adsorbed proteins from nanocomposite scaffolds surface to SDS solution, 1 mL of aqueous solution of sodium dodecyl sulfate (SDS, 1 wt. %) was added followed by shaking of TCP for 60 min. The amount of protein adsorbed (μg/cm²) on the surface was evaluated from the concentration of protein in the SDS solution employing a protein analysis kit (Micro BCA Protein Assay Kit, Pierce Biotechnology, IL, USA) [28]. The bovine serum albumin standard solution included with the test kit was used to calibrate protein concentration. Data were expressed as the means ±SD of three independent measurements.
5.3.8.2 Haemolysis test

*In vitro* erythrocytes lysis test was performed as a preliminary toxicity test assessed by determining the haemoglobin released as a result of membrane leakage or disruption caused by exposure to low doses of the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites [29]. Briefly, fresh blood obtained from a healthy rabbit was collected in anticoagulant solution (ethylene diamine tetraacetic acid) and centrifuged at 1000 x g for 10 min at 4 °C. The buffy coat and plasma were discarded followed by the dilution of washed erythrocytes with isotonic buffer (20 mM PBS) to prepare 50% hematocrit. The extent of haemolysis was studied by incubating the RBC suspension with various concentrations (10 to 50 μg/ml) of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites at 37 °C for 1 h. The incubated solutions were centrifuged at 1500 x g after 1 h and supernatant was collected and analyzed by ultraviolet-visible spectroscopy (λ<sub>max</sub> = 576 nm) for released haemoglobin. The percentage hemolysis was determined by the following equation:

\[
\text{Hemolysis (\%)} = \frac{\{\text{Abs}(t) - \text{Abs}(c)\}}{\{\text{Abs}(100\%) - \text{Abs}(c)\}} \times 100
\]

where,

\[\text{Abs}(t) = \text{Absorbance of the supernatant from samples incubated with the nanocomposites.}\]
\[\text{Abs}(c) = \text{Absorbance of the supernatant from controls (normal saline).}\]
\[\text{Abs}(100\%) = \text{Absorbance of the supernatant of controls incubated in the presence of 1\% Triton X-100, which causes complete lysis of RBCs (total lysis).}\]

5.3.8.3 Platelet adhesion

To form Platelet-rich plasma (PRP), the whole blood was centrifuged at a rate of 1000 rpm/min for the time period of 15 minutes. The PRP was then overlaid on the surface of the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites and incubated at 37 °C for 1 h. To remove the non-adherent platelets after incubation, all the nanocomposites samples were rinsed with PBS buffer several times. The adhered platelets were fixed in 2.5% glutaraldehyde solution at room temperature for 1 h that
is followed by dehydration in a gradient ethanol/distilled water mixture (from 30% till 90%) for 15 min each and dried. The surfaces of platelet attached on the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites were then observed by SEM [30].

5.3.9 Alkaline phosphatase activity (ALP) assay

Osteoblast cells were isolated from femur bone shaft of the Wistar rat. The medium consisted of Dulbecco’s modified Eagle medium (DMEM; Gibco-BRL, Life Technologies, Grand Island, NY), supplemented with 15% fetal bovine serum (FBS; Gibco-BRL, Life Technologies, Grand Island, NY) and 100g/mL penicillin–streptomycin (Gibco-BRL, Life Technologies). The cell suspensions were seeded on n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites samples and cultured in polystyrene 6-well dishes. Non-adherent cells were removed from the cultures after 4 days by rinsing the wells with phosphate buffered saline (PBS) washes and subsequent medium changes. Adherent cells were expanded as monolayer cultures in a 5% CO₂/95% air atmosphere at 37 °C with medium changed every 3 days. The confluent cells were dissociated with trypsin/EDTA 0.05% and sub-cultured in new 6-well culture dishes at a plating density of 5×10⁵ cells/dish. Cell loaded samples were incubated in 15% FBS supplemented DMEM for 3 days. At various time periods, activity of alkaline phosphatase enzyme was assayed following method as described elsewhere [31,32]. ALP activity was determined by an assay based on the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol. About 50 μL of the Triton lysate was added to 125 μL of active reagent containing 0.012 M p-nitrophenyl phosphate in 0.05 M diethanolamine, pH 9.8, and incubated for 30 minutes at 37 °C. The reaction was stopped with 50 μL of 2.5 M sodium hydroxide and the ALP activity was determined by measuring the absorbance of p-nitrophenol at 405 nm using a Microplate Reader (Bio-Rad). Alkaline phosphatase activity was normalized and expressed as the total protein content (U/mg protein).

5.4 Results and Discussion

The nanocomposite scaffolds, n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT, n-HA/β-CD/CS-LT were prepared at room temperature, high temperature and low temperature, respectively and were compared with the binary nanocomposite (CS/n-HA) system. The optimization of the different properties of n-HA/β-CD/CS and the role played by individual component present in the nanocomposite scaffold to endow
structural stability, integrity, apatite nucleation, mechanical strength, hemocompatibility and biocompatibility have been investigated using various physico-chemical and biological techniques.

5.4.1 TEM

The comparative study of TEM micrographs of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites shown in Figure 2, revealed a rigorous agglomeration of particles in case of CS/n-HA nanocomposite contrary to appreciably homogeneous distribution of particles in n-HA/β-CD/CS-LT nanocomposite with needle shaped morphology as compared to n-HA/β-CD/CS-(RT,HT) indicating that the presence of β-CD hampered the aggregation of CS/n-HA particles in n-HA/β-CD/CS at low temperature [33]. The average size of the needle shaped particles of n-HA/β-CD/CS-LT was found to be in the range of ~10-12 nm as against the average size of n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT (~17-19 nm) and CS/n-HA (~22-40 nm) particles. Therefore it is inferred that the incorporation of β-CD in CS/n-HA possibly limit the size of the particles particularly at low temperature where the needle shaped particle are relatively more prominent and dispersed.

5.4.2 SEM

The SEM images have been recorded to further study the morphological detail of the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite systems. A comparative study of SEM micrographs of n-HA/β-CD/CS-(RT,HT,LT) with that of CS/n-HA nanocomposite system as shown in Figure 3(a-d), highlighted a relatively highly porous surface with good interconnectivity for n-HA/β-CD/CS-(RT,HT,LT) as compared to smoother and even surface in case of CS/n-HA system.
These results suggested that the addition of β-CD influenced the surface morphology of CS/n-HA which seems to be an important requirement for the tissue in-growth, bone formation and biological fixation with surrounding tissue [7,8]. The morphological changes may be understood in terms of possible interaction of OH of β-CD with Ca$^{2+}$ of n-HA and with NH$_2$ of CS in n-HA/β-CD/CS nanocomposites leading to changes in physical properties [34,35]. However it is evident from Figure 3(d), that n-HA/β-CD/CS-LT showed relatively more rough and porous surface comparative to n-HA/β-CD/CS-(RT,HT) nanocomposites which has also been evident from the AFM analysis observations.

Figure 2: Representative TEM Micrographs of CS/n-HA (a) and n-HA/β-CD/CS-RT (b) -HT (c) -LT (d).
5.4.3 \textit{In-vitro} bioactivity evaluation of n-HA/β-CD/CS nanocomposite scaffolds

It is a common criterion that the \textit{in-vitro} calcification efficiency of biomaterials has coordination with the bone-bonding capability \textit{in-vivo}. Bioactivity is an important concern in the chemical interactions between the implant materials and bone tissue, ultimately affecting the \textit{in-vivo} success of bone grafting materials. The chemical reactions occurring at the surface of a material exposed to body fluids in order to form a surface layer of hydroxyl carbonated apatite upon implantation results in bioactivity which is a fundamental principle for establishing bonding with natural bone. Hence, investigating the biological behavior of biomaterial nanocomposites in SBF is believed to be the most efficient method to authenticate their bioactivity in the body environment. The \textit{in-vitro} bioactivity of the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites soaked in SBF solution for the time period of 15 and 30 days, has been monitored by SEM microphotograph [Figure 3(a1-d2)]. The micrographs revealed that there was significant biomimetic deposition of HA in all the three matrix surfaces comparative to CS/n-HA nanocomposite. However, a comparative SBF study for the time period of 30 days revealed a long range deposition of HA on the surface of the n-HA/β-CD/CS-HT nanocomposite [Figure 2(c2)] as compared to short range deposition of HA in the form of small dots scattered on the entire surface of n-HA/β-CD/CS-RT [Figure 3(b2)] whereas the n-HA/β-CD/CS-LT matrix exhibited relatively longer range of deposition of thick mineral layer showing full coverage over the whole surface indicating faster deposition of apatite which has also been confirmed by EDX observations that may be attributed to its relatively rougher surface [Figure 3(d2)]. The higher apatite formation in case of n-HA/β-CD/CS-(RT,HT,LT) compared to CS/n-HA can be explained in terms of the mineralization inducing capability of β-CD responsible for the nucleation and growth of bone-like apatite onto biomaterials, that is linked with uptake of calcium and phosphate ions from the physiological environment [Figure 4]. It may be concluded that n-HA/β-CD/CS-(RT,HT,LT) nanocomposite possess promising potential mineralization ability facilitating osteointegration \textit{in-vivo} and bone ingrowth formation [36,37].
Figure 3: Characteristic SEM images of CS/n-HA (a) n-HA/β-CD/CS-RT (b) n-HA/β-CD/CS-HT (c) n-HA/β-CD/CS-LT (d) nanocomposites and their respective SBF study after 15 days(a1-d1) and 30 days (a2-d2).
5.4.4 Energy Dispersive X-ray Spectroscopy (EDX)

The comparative study of EDX spectra of n-HA/β-CD/CS-(RT,HT,LT), CS/n-HA and their respective SBF scaffolds kept for 15 and 30 days has been made [Figure 5(a-d2)]. The EDX analysis clearly show the presence of Calcium (Ca) and Phosphorous (P) contents in the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites. However it can be noticed that Ca and P contents found in greater amount in case of n-HA/β-CD/CS-LT [Figure 5(d2)] further confirming the faster deposition of apatite on its surface after exposure to SBF relative to n-HA/β-CD/CS-(RT,HT) as inferred from SEM result. The observed semiquantitative ratio of Ca/P of 1.78 against the expected range of 1.67±0.67 in natural bone has been found in the EDX spectra of n-HA/β-CD/CS-LT nanocomposite scaffold kept in SBF for 30 days [38] as compared to the values of 1.85, 1.72 and 1.50 obtained in case of n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT and CS/n-HA nanocomposite scaffolds, respectively kept in SBF for the same period of time, indicating that the phase and composition are similar to the mineral phase of the bone. The results of EDX mapping showed presence of C, O, Ca and P elements in all the nanocomposite systems however, in general, indicated greater deposition of C, O, Ca and P in case of n-HA/β-CD/CS-(RT,HT,LT) compared to CS/n-HA nanocomposite. The EDX elemental mapping of CS/n-HA and n-HA/β-CD/CS-LT has been shown in Figure 6, represented the relatively high and homogeneous deposition of the key components of apatite, i.e., Ca and P in case of n-HA/β-CD/CS-LT compared to CS/n-HA nanocomposite system. Consequently, it can be concluded that tailoring β-CD into the polymer matrix may translate into an effective method to adjust the apatite-like formation in these biomaterials, enhancing their cell response for optimal application performance.

Figure 4: Mineralization of hydroxyapatite on n-HA/β-CD/CS nanocomposite immersed in SBF.
Figure 5: EDX Spectra of CS/n-HA (a) n-HA/β-CD/CS-RT (b) n-HA/β-CD/CS-HT (c) n-HA/β-CD/CS-LT (d) nanocomposites and their respective SBF study after 15 days(a1-d1) and 30 days(a2-d2).
Figure 6: EDX Elemental [C, O, Ca, P] mapping of CS/n-HA and n-HA/β-CD/CS-LT nanocomposites.
5.4.5 FTIR analysis

The possible interaction among the various components in n-HA/β-CD/CS nanocomposite has been displayed graphically in Scheme 1. The fundamental information regarding the interaction of various components in CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites has been obtained by comparing the FTIR spectra [Figure 7]. The FTIR spectra of CS/n-HA exhibited the characteristic bands of chitosan and nano-hydroxyapatite [7,8]. The FTIR spectra of n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT and n-HA/β-CD/CS-LT nanocomposites exhibited bands characteristic of n-HA, CS and β-CD moieties. In the FTIR spectra of n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT and n-HA/β-CD/CS-LT, the presence of HA in β-CD-CS matrix can be identified by its representative bands of phosphate group in the range of 418-454, 502-566 and 603-610 cm\(^{-1}\) respectively assigned to phosphate bending modes of vibrations. However, the stretching mode of vibration of phosphate group in n-HA overlaps with the C-O-C stretching vibration of CS and -C-O- band of β-CD discerns as a broad band in the region of 1035 cm\(^{-1}\)-1038 cm\(^{-1}\), while OH stretching band of HA gets overlapped with the OH stretching band of CS and β-CD and a broad peak appeared in 3390-3500 cm\(^{-1}\) region [39,40,36]. The new bands in the range of 2320-2355 cm\(^{-1}\) in all the three n-HA/β-CD/CS-(RT,HT,LT) nanocomposites may be attributed to the coordinative nature of interaction between –OH of β-CD and Ca\(^{2+}\) of n-HA that becomes the basis of nucleation and growth point of apatite crystals [41,33]. The peak at ~1650 cm\(^{-1}\) may be due to carbonyl stretching mode of secondary amide moiety which confirmed the interaction between CS and β-CD [39]. Thus it may be concluded from FTIR spectra that all the three components in n-HA/β-CD/CS nanocomposites prepared at different temperatures involves the expected interactions among different components.
Scheme 1: Possible interaction between different components in n-HA/β-CD/CS nanocomposite.

Figure 7: FTIR spectra of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites.
5.4.6 TGA-DTA analysis

It is essential to verify the thermal stability via TGA analysis not just in the temperature limit of human body but also in higher temperature intervals that comprises of sterilization processes in order to meet out the biocompatibility of the biomaterials. The TGA and DTA curves of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) have been displayed in Figure 8 and Figure 9, respectively. The TGA graph of CS/n-HA nanocomposite involves two step weight loss in the range of 90-150 °C and 200-410 °C in consistency with DTA curve with total weight loss of ~70-72%, indicating that the n-HA raised the thermal stability of chitosan [7-8]. The thermal stability of CS/n-HA nanocomposite got further increased by induction of β-CD in CS/n-HA resulting in n-HA/β-CD/CS-(RT,HT,LT) nanocomposites revealed by two step weight loss corresponding to the moisture loss and decomposition of β-CD, respectively in the temperature range of 100-120 °C and 290-380 °C. This has been further confirmed by DTA curves with total weight loss in the range of (25-40) %. Thus comparative study of thermal analysis of these nanocomposites shows that thermal stability varies as n-HA/β-CD/CS-LT> n-HA/β-CD/CS-HT> n-HA/β-CD/CS-RT > CS/n-HA in parity with comparative weight loss suggesting that β-CD fairly raised the thermal stability of CS/n-HA nanocomposite in n-HA/β-CD/CS-(RT,HT,LT) possibly due to regular increased interactions [42,39,8].

![Figure 8: Thermogravimetric analysis (TGA) for CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites.](image-url)
5.4.7 X-ray diffraction (XRD) studies

The comparative XRD patterns of the resulting nanocomposites including original human bone (a), CS/n-HA (b), n-HA/β-CD/CS-RT (c), n-HA/β-CD/CS-HT (d) and n-HA/β-CD/CS-LT (e), are shown in Figure 10 (i),(a-e). The average crystallite sizes of the human bone and nanocomposites were calculated using the FWHM as calculated by Scherer equation [4].

\[ L = K \frac{\lambda}{\beta \cos \theta} \]

\( \lambda \) is the wavelength of monochromatic X-ray beam radiation Cu radiation (\( \lambda = 1.5406 \) Å), \( \theta \) is the peak diffraction angle (Bragg’s angle), \( K \) is a Scherrer constant defined as the crystallite shape and is approximately equal to 0.9. The characteristic peaks of n-HA expected to appear at \( 2\theta = 26, 29.3, 32.3 \) etc. were observed in CS/n-HA (b), n-HA/β-CD/CS-RT (c), n-HA/β-CD/CS-HT (d) and n-HA/β-CD/CS-LT (e) confirming the presence of n-HA crystallites which matches well with the XRD peaks of apatite present in the original human bone displayed in Figure 10(i).a. This indicates that crystallization of n-HA still existed after nanocomposite formation which may be resulted from interfacial binding between n-HA particles and polymers matrices.
The disappearance of characteristic peaks corresponding to chitosan in CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites may reasonably be due to possible interaction of CS with n-HA and β-CD. However the appearance of a new crystalline peaks at 2θ = 12.95 and 14.5 confirmed the presence of β-CD in n-HA/β-CD/CS-(RT,HT,LT) nanocomposites [Figure 10(i).c-e] [34]. The average crystallite sizes of 25.4, 17.5, 19.8 and 12.9 nm for CS/n-HA, n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT and n-HA/β-CD/CS-LT nanocomposites, respectively affirm their nano-architecture comparable with the average size of 14.7 nm observed in human bone. The XRD patterns of original human bone (a), CS/n-HA (b), n-HA/β-CD/CS-RT (c) n-HA/β-CD/CS-HT (d) and n-HA/β-CD/CS-LT (e) after 30 days SBF study, Figure 10(ii).a-e indicated the obvious presence of hydroxyapatite peaks. The low crystallinity of the newly formed hydroxyapatite crystals is responsible for the slight broadness of their diffraction peaks in the XRD pattern. The appearance of β-CD diffraction peaks in n-HA/β-CD/CS-(RT,HT,LT) got noticeably diminished after soaking in SBF for 30 days suggesting the higher growth of apatite layer further complimenting the SEM results.

5.4.8 Swelling Test

The comparative swelling ratio study of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites in SBF solution for different time intervals (1, 7, 14, 21 and 28 days), Figure 11 revealed that there has been significant increase in swelling ratio of all n-HA/β-CD/CS-(RT, HT, LT) nanocomposites as compared to CS/n-HA which may be advocated in terms of polyhydroxyl nature of β-CD that makes room for the enhanced hydrogen bonding of the resulting system leading to increased interactions between n-HA, CS and β-CD. However, it is observed that n-HA/β-CD/CS-LT displayed superior swelling capability compared to n-HA/β-CD/CS-(RT, HT). Thus it may be concluded that the medium uptake ability of the CS/n-HA nanocomposite got relatively more enhanced with the incorporation of β-CD at low temperature that would support diffusion of medium and nutrients into the nanocomposite scaffold [44].
Figure 10: XRD patterns of (a) Human Bone (b) CS/n-HA (c) n-HA/β-CD/CS-RT (d) n-HA/β-CD/CS-HT (e) n-HA/β-CD/CS-LT (i) and their respective SBF spectra after 30 days (ii).
5.4.9 Biodegradation *in-vitro*

According to tissue engineering approach, it is imperative for a scaffold to be considered as ideal material, it should degrade simultaneously as new tissue formation takes place. In order to authenticate the degradability of the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite scaffolds, the degradation study was performed for 28 days in the PBS + lysozyme medium. The n-HA/β-CD/CS-LT nanocomposite was degraded up to 28.9% of its total weight after incubation period of 28 days while the degradation (%) for n-HA/β-CD/CS-RT, n-HA/β-CD/CS-HT and CS/n-HA was found to be 27.9%, 26.7% and 17.9% respectively [Figure 12]. The better degradation can be understood in terms of breakdown of glycosidic linkage present in CS and β-CD by lysozyme. Therefore from the degradation studies, it may be concluded that β-CD when fused with CS/n-HA in the series of n-HA/β-CD/CS-(RT,HT,LT) has improved the degradation ability of the synthesized nanocomposites in the physiological conditions [45].
Figure 12: Biodegradation study of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites after designed days.

5.4.10 In-vitro cytotoxicity assay of nanocomposites

The assay is based on scrutinizing the ability of living cells to metabolize a water-soluble tetrazolium yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into insoluble purple formazan salt. The nanocomposites scaffolds n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA were subjected to cytotoxic studies with human osteoblasts MG-63 cells. The cells were incubated with various concentrations (0-128µg/ml). The incubation of n-HA/β-CD/CS-(RT,HT,LT) nanocomposites for 24 hrs revealed substantially enhanced metabolic activity of the cells compared to CS/n-HA nanocomposite indicating that the n-HA/β-CD/CS-(RT,HT,LT) nanocomposites do not seem to induce undesired cellular toxic effects [Figure 13]. However, interestingly, over 80% cells viability has been observed in case of n-HA/β-CD/CS-LT at all investigated concentrations compared to n-HA/β-CD/CS-(RT,HT) nanocomposite samples indicating relatively superior biocompatible nature of n-HA/β-CD/CS-LT confirming that the incorporation of β-CD in CS/n-HA matrix at low temperature may be a promising candidate for bone tissue engineering [46,40].
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5.4.11 Blood compatibility tests

The protein adsorption study on nanocomposite scaffold is significant as the cell and material interaction initiate with protein adsorption further affecting the cell adhesion and proliferation [47]. Therefore, the in-vivo potential of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite samples was further confirmed by in-vitro results of protein adsorption study, haemolytic activity and platelet adhesion [Figure 14(a-c)].

The amount of protein adsorbed on the surface of different nanocomposite scaffolds viz., CS/n-HA, n-HA/β-CD/CS-(RT,HT,LT) and tissue culture plate, has been evaluated by incubating the samples with FBS. All nanocomposite scaffolds showed better protein adsorption as compared to TCP used as control [Figure 14(a)]. The nanocomposite scaffolds, n-HA/β-CD/CS-(RT,HT,LT) exhibited significantly better average protein adsorption value (170±9.67 μg/cm², 110±9.67 μg/cm², 90±0.50 μg/cm², respectively) in comparison to CS/n-HA nanocomposite scaffold which may possibly be due to enhanced hydrophobicity and roughness imparted by β-CD in CS/n-HA nanocomposite supporting protein adsorption [33], however, n-HA/β-CD/CS-LT nanocomposite scaffold exhibited a slightly better protein adsorption as compared to n-HA/β-CD/CS-(RT,HT) owing to maximum RMS roughness as
obtained by AFM analysis [48]. In addition, for materials that come into contact with blood, the formation of clot is the most undesirable though frequently occurring event that has a tremendous influence on subsequent bone healing phenomenon in the peri-implant healing compartment restricting the clinical acceptance of a material to be used as biomaterial [49]. A significant difference in hemolysis percentage could be observed in case of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite samples while the least haemolytic rate was found to be in case of n-HA/β-CD/CS-LT, however the hemolysis rates for all the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite samples stayed much lower than 5%, which is regarded as a biosafety threshold and stands for well-behaved hemocompatibility [Figure 14(b)]. On the other hand, a large amount of platelets was aggregated and accumulated on CS/n-HA nanocomposite sample compared to n-HA/β-CD/CS-(RT,HT,LT) samples [Figure 14(c)]. This indicates thrombus formation might be more likely on the surface of CS/n-HA nanocomposite which may possibly be explained on the platelet adhesive and activating nature of chitosan [50] while in case of n-HA/β-CD/CS-LT nanocomposite sample, it could be seen that relatively less number of platelets adhering to the surface which appeared to be nearly round with only one or two short pseudopodia, implying a negative activation in comparison to nanocomposite scaffolds synthesized at RT and HT further suggesting that addition of β-CD in CS/n-HA matrix at low temperature resulted into a relatively better hemocompatible system supporting the hemofriendly property of β-CD [17,33]. Our findings indicated that among n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites, n-HA/β-CD/CS-LT showed relatively better hemocompatibility and thus expected to be employed for bone implant applications.

5.4.12 ALP activity

Alkaline phosphate (ALP) is an important feature of osteoblast cells expressed in their differentiation phase and a noteworthy quantitative marker of osteogenesis [51]. The *in-vitro* ALP activity of osteoblast cells cultivated with n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites was investigated at 1,4 and 8 days. As shown in Figure 15, ALP activities of n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites got increased with time. However, no significant difference in ALP activity has been observed for n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites for 1 and 4 days. While in general, a higher ALP activity was observed for n-HA/β-CD/CS-
(RT, HT, LT) nanocomposites after culturing for 8 days compared to CS/n-HA nanocomposite where a highest ALP activity was noticed for n-HA/β-CD/CS-LT may be due to relatively more rougher surface triggering an upregulation of ALP, correlated with the first check-point for osteogenic differentiation [30]. Therefore, it can be concluded that the participation of biocompatible β-CD in CS/n-HA matrix provided an effective substrate for cellular differentiation possibly attributable to its HA mineralization property that improved the nucleation of HA, resulting in the enhancement of osteogenesis [52].

Figure 14: Haemocompatibility assessment: (a) Protein adsorption Study (b) Haemolysis (%) (c) SEM observations of Platelet adhesion study; of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites. *Statistical significance level by t-test (p < 0.05).
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5.4.13 Atomic Force Microscopy (AFM) analysis

AFM derived surface topographies for the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites are illustrated in Figure 16. The distinctly rougher and denser surfaces have been observed for the n-HA/β-CD/CS-(RT,HT,LT) nanocomposites which possibly promote protein adsorption and cell proliferation as compared to smoother surface of CS/n-HA nanocomposite [22,28]. A comparative RMS study to evaluate the average surface roughness of ternary nanocomposites n-HA/β-CD/CS-(RT,HT,LT) exhibited a significant rise in the RMS roughness values as compared to the CS/n-HA nanocomposite with a highest value of 17.4 nm rms roughness for n-HA/β-CD/CS-LT nanocomposite which may be explained in terms of added participation of β-CD in CS/n-HA at low temperature.

5.4.14 MIC and MBC assessment

The comparative antibacterial activity results suggested that all the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposite scaffolds exhibited antibacterial properties for both Gram positive and Gram-negative bacteria due to the presence of antibacterial property offered by CS and β-CD [53,17]. However it is found that MIC and MBC values for n-HA/β-CD/CS-LT were comparatively lower than the values
shown by n-HA/β-CD/CS (RT,HT) and CS/n-HA [Table 1]. The superior antibacterial nature of n-HA/β-CD/CS-LT may be due to relatively smaller particle as compared to size of n-HA/β-CD/CS (RT,HT) and CS/n-HA that may be explained by relating higher bacterial interaction ability of small sized particles with increased surface area to volume ratio leading to greater disruption of cell membranes and destruction of cytoplasm [54].

5.4.15 Mechanical Properties

5.4.15.1 Shore hardness and compressive strength of nanocomposites

The initial mechanical features are generally significant in choosing the nanocomposite scaffold materials for bone tissue engineering. Shore hardness is a kind of dynamic hardness that measures the height of the bounce of a diamond tipped hammer on the sample to be analyzed dropped from a fixed height [8]. A comparative study of shore hardness of CS/n-HA and n-HA/β-CD/CS-HT, n-HA/β-CD/CS-LT, performed on their pellets revealed the average value of shore hardness of 45±3.22, 70±2.88, 74±2.90 and 88±2.78, respectively [Table 2]. The highest shore hardness value of n-HA/β-CD/CS-LT nanocomposite relative to CS/n-HA and n-HA/β-CD/CS(RT,HT) may be explained possibly in terms of higher interaction displayed between CS/n-HA and β-CD at low temperature. In addition, the compression test has been extensively accepted and used effectively for characterization of mechanical properties of natural bone. The samples were made in the form cylindrical pellets of 6 mm diameter and 12 mm height in agreement with the compression mechanical test guidelines set in American Standard Test and Measurement (ASTM F 451-95) [21].

The results of average compressive modulus revealed that CS/n-HA showed extremely lower compressive modulus [273.8 MPa] relative to the values obtained for n-HA/β-CD/CS-(RT,HT), i.e., 811.52714 and 899.99951 MPa, respectively, comparable to the values observed for human trabecular bone [55] while the average compressive modulus value of n-HA/β-CD/CS-LT was found to be approximately five fold greater [5,703.2 MPa].
Figure 16: Atomic force 2D scan micrographs of n-HA/β-CD/CS-LT (a) n-HA/β-CD/CS-RT (b) n-HA/β-CD/CS- HT (c) and CS/n-HA nanocomposites (d) and their respective Root mean square (RMS) roughness (scan size= 10μm, scan rate =2 Hz). *Statistical significance level by t-test (p < 0.05).
Table 1: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of n-HA/β-CD/CS-(RT, HT, LT) and CS/n-HA upon the studied bacteria (Values in μg/mL).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>CS/n-HA</th>
<th>n-HA/β-CD/CS-RT</th>
<th>n-HA/β-CD/CS-HT</th>
<th>n-HA/β-CD/CS-LT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>Gram +ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>1000</td>
<td>2000</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>256</td>
<td>512</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>Gram –ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>1600</td>
<td>3200</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>V. cholera</td>
<td>256</td>
<td>512</td>
<td>128</td>
<td>256</td>
</tr>
</tbody>
</table>

Similarly the compressive strengths [Table 2] varied as n-HA/β-CD/CS-LT>n-HA/β-CD/CS-RT>n-HA/β-CD/CS-HT>CS/n-HA suggesting that addition of β-CD in CS/n-HA at LT has significantly increased the hardness and modulus clearly showing that mechanical properties were dependent on reaction temperature [22,53]. The increase in mechanical strength parameters of n-HA/β-CD/CS-(RT, HT, LT) may be explained in terms of the enhanced intermolecular hydrogen bonding between n-HA, β-CD and CS moieties that increased the cross-linkage and thus decreased the molecular mobility and the free volume present in the nanocomposite system imparting more rigidity to the system [56] however, homogeneous distribution of particles in case of n-HA/β-CD/CS-LT lead to remarkable improvement in the mechanical properties parameters orthopedic applications maintaining sufficient integrity [57], suggesting that n-HA/β-CD/CS-LT nanocomposite may find potential use in Bone tissue engineering.
Table 2: Mechanical properties of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites and their pellets display.

<table>
<thead>
<tr>
<th>Nanocomposite Samples</th>
<th>Compressive Strength [MPa]</th>
<th>Compressive Modulus (Automatic) [MPa]</th>
<th>Shore Hardness</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/n-HA</td>
<td>15.56611</td>
<td>273.8735</td>
<td>45±3.22</td>
</tr>
<tr>
<td>n-HA/β-CD/CS-RT</td>
<td>39.50298</td>
<td>*811.5271</td>
<td>*70±2.88</td>
</tr>
<tr>
<td>n-HA/β-CD/CS-HT</td>
<td>39.40665</td>
<td>*899.9995</td>
<td>*74±2.90</td>
</tr>
<tr>
<td>n-HA/β-CD/CS-LT</td>
<td>*158.01335</td>
<td>*5,703.2533</td>
<td>*88±2.78</td>
</tr>
</tbody>
</table>

5.5 Conclusion

In the present work, we make an attempt to synthesize a novel ternary nanocomposite system by successfully incorporating cycloheptaglucan/β-cyclodextrin with nano-hydroxyapatite and chitosan system by a simple co-precipitation method at three different temperatures to investigate various physico-chemical, biological properties and mechanical properties for the potential use in orthopaedic applications. The FTIR spectra of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposite scaffolds exhibited the bands characteristic of organic and inorganic moieties indicating significant intermolecular interaction between the different components in all the three nanocomposites. The comparative XRD patterns of the n-HA/β-CD/CS-(RT,HT,LT) and CS/n-HA nanocomposites revealed that the size of n-HA/β-CD/CS-(RT,HT,LT) decreased in general upon incorporation of β-CD while the size of n-HA/β-CD/CS-LT was found to be smallest. The comparative TGA/DTA results, inferred, an increase in thermal stability of n-HA/β-CD/CS-(RT,HT,LT) nanocomposite compared to CS/n-HA nanocomposite. The morphology of CS/n-HA nanocomposite has also been influenced by addition of β-CD inducing roughness with highest degree in n-HA/β-CD/CS-LT nanocomposite as revealed by comparative analysis of SEM and AFM results. The comparison of *in-vitro* bio-mineralization
studies monitored by SEM images of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites suggested the best apatite forming ability of n-HA/β-CD/CS-LT leading to superior ability for bone ingrowth and possibility of good osteointegration in-vivo. The MTT and ALP assay studies on CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) nanocomposites revealed highest cell proliferation in n-HA/β-CD/CS-LT nanocomposite compared to n-HA/β-CD/CS-(RT,HT) and CS/n-HA nanocomposites warranting advanced non-toxicity and a significant role in cell differentiation and initiation of mineralization process. The comparative results of antibacterial activity against both Gram-positive and Gram-negative bacteria and blood compatibility assessment of CS/n-HA and n-HA/β-CD/CS-(RT,HT,LT) conveyed an improved antibacterial property of n-HA/β-CD/CS-LT nanocomposite. The considerable improvement in the values of shore hardness and compressive strength of the n-HA/β-CD/CS-LT nanocomposite compared to CS/n-HA and n-HA/β-CD/CS-(RT,HT) indicated relatively increased interactions. Given these findings, this study is the first demonstration of standalone synthesis and application of the bioactive n-HA/β-CD/CS-(RT,HT,LT) nanocomposite systems, the detailed study of which showed that the n-HA/β-CD/CS-LT nanocomposite may find excellent possible applications for various implantable devices in orthopedic applications to increase their osseointegration and to limit their rejection in the field of Bone tissue engineering. Thus it may be concluded that the validation of such simple and bioactive nanocomposites is crucial for the clinical translation of tissue-engineered materials for bone repair.
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Chapter 5: 

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