CHAPTER 4
NANO-HYDROXYAPATITE/CHITOSAN-STARCH NANOCOMPOSITE AS A NOVEL BONE CONSTRUCT: SYNTHESIS AND IN-VITRO STUDIES
4.1 Introduction

In continuation to our previous work on polymer nanocomposites [1,2], an attempt has been made to synthesize a biomaterial significant in the field of bone tissue engineering employing renewable, bioactive and naturally abundant polymers [3]. The bone repair is a prevalent and challenging clinical issue in orthopedic surgery. In the fast changing dynamics of the world order, a large number of people being afflicted with bone defects these days. The autogenic and allogenic procedures are commonly in practice to deal with bone defects. It has been observed that autogenic bones reduce the risk of immune rejection though involve multiple surgeries and associated with donor site morbidity while allogenic bones bear risk of infections and immune feedbacks. Therefore it has been realized to design and develop the materials that can serve as efficacious bone grafts substitutes and as artificial prosthesis to address the patient requirements [4,5]. In view of the serious limitation in traditional therapies, tissue engineering provides a novel platform in bone reconstruction incorporating therapies that mimic the critical aspects of natural biological processes.

Bone is the most typical triphasic calcified tissue in mammals that consist of cellular components, hydrated extracellular organic matrix and extracellular inorganic phase which is chiefly a major reservoir for calcium and phosphate ions needed for diverse metabolic activities. It has a wide spectrum of mechanical properties, depending on its type, humidity, density, porosity, mineral content and interfacial bonding between constituents. Hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\), (HA) is the fundamental inorganic component of bone and is a biologically active calcium phosphate ceramic that is employed in surgery to replace and mimic bone. The shape of HA crystals in a natural bone is needle like or rod–like with length and width of 40-60 nm and 10-20 nm, respectively [6-9]. Several reports appeared regarding the synthesis of nanometer size HA(n-HA) having various shapes viz. the needle like HA has been synthesized by organic gel system and homogeneous precipitation while rod like HA has been synthesized by precipitating calcium nitrate tetrahydrate and ammonium dibasic phosphate in the presence of polyacrylic acid followed by hydrothermal treatment [10]. The HA thus formed displayed bone-bonding properties, has been extensively used in hard tissue replacement in view of their biocompatibility and osteoconductivity features, but the brittleness of HA materials limits its application in bone tissue engineering. Therefore an intense and immediate development of
nanocomposite materials with controllable bioactivity and satisfactory mechanical properties for bone tissue engineering is demanded, making this field a fertile research area and attempts have been made to overcome this problem by combining HA with different polymeric additives such as poly(vinyl alcohol), poly(lactic acid), poly(acrylic acid) etc.

In search of environmentally favorable material from natural and renewable resources as an alternative for bone tissue replacement, the researches have been directed towards the combination of HA with natural polymers in the past few years such as starch which has garnered interest due to its attractive fusion of availability, low price and biocompatibility proving it a potential material in medicine, agriculture and packaging industries [11-14]. On the other hand Chitosan (CS) which is a randomly partially deacetylated form of chitin, the β(1,4)-linked polymer of N-acetyl-D-glucose-2-amine, extracted from crustaceans exoskeletons, has been adopted in different biomedical researches such as bone tissue engineering, nerve, retinal tissue engineering, drug carriers and blood vessels, is a biocompatible polymer that can be degraded by enzymes in human body leading to non-toxic degradation products [15-20].

While starch being polar, dissolves in ecofriendly solvent, water, is composed of linear amylose (poly-α-1,4-D-glucopyranoside) and branched amylopectin (poly-α-1,4-D-glucopyranoside and poly-α-1,6-D-glucopyranoside) [21]. The OH group on starch framework may possibly be the facile centers for interaction with amino groups of CS and Ca\(^{2+}\) ions of HA facilitating crystallization process of n-HA [6].

Herein we report the synthesis of n-HA/CS-ST and n-HA/CS nanocomposite systems via co-precipitation approach, their characterization and a comparative study of n-HA/CS-ST against n-HA/CS nanocomposite in terms of crystallinity, antibacterial activity, mechanical and thermal stability. The in-vitro evaluation of the physical and biological properties of the proposed nanocomposite n-HA/CS-ST has been done after immersion in simulated body fluid.

4.2 Experimental

4.2.1 Materials and Methods

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) (MTT), Chitosan (CS) with degree of deacetylation (>85%) and Dulbecco’s modified Eagle’s medium
(DMEM) were purchased from Sigma-Aldrich (USA) and Invitrogen, USA, respectively. Extra pure water soluble wheat starch, [Ca(NO\(_3\)]\(_2\)-4H\(_2\)O] (99%), (NH\(_4\))\(_2\)HPO\(_4\) (DAHP) (99%), CH\(_3\)COOH (99.8%), NaOH (>97%), DMSO, NaCl, NaHCO\(_3\), KCl, K\(_2\)HPO\(_4\),3H\(_2\)O, MgCl\(_2\)-6H\(_2\)O, CaCl\(_2\), Na\(_2\)SO\(_4\),tri-(hydroxymethyl) aminomethane [TRIS], HCl and ammonia solution (25%) have been procured from Merck, Mumbai, India. All chemicals were used without further purification. Double distilled water was used in all the experiments.

### 4.2.2 Synthesis of n-HA/CS-ST nanocomposite

The synthesis of n-HA/CS-ST nanocomposite was carried out via co-precipitation approach at room temperature. A solution of starch (2gm) prepared in 100 ml of distilled water was slowly added to (2 gm) solution of CS dissolved in 100 ml of 2 wt% aqueous acetic acid. The mixture was kept on magnetic stirring at 1200 rpm at room temperature until the contents were thoroughly mixed. This was followed by addition of 0.1 M [Ca(NO\(_3\)]\(_2\)-4H\(_2\)O] and 0.3 M DAHP solutions in drop-wise manner to CS-ST mixture kept on stirring maintaining Ca/P stoichiometric ratio of 1.67. The overall mixture turned opaque and the pH of the mixture was adjusted to about ~11 by using 0.5 M NaOH solution in order to accelerate the nucleation of n-HA expected at high pH value leading to a milky white product which ultimately changed to creamish white material on constant stirring for 24 hours. The product thus obtained was allowed to ripe for another 24 hours without stirring. The product settled on ageing, was filtered and washed several times with distilled water until the filtrate became neutral. The product thus isolated was dried in oven at 85 °C. The synthesis of n-HA/CS nanocomposite was also carried out adopting the similar approach to compare the various properties of n-HA/CS-ST and n-HA/CS nanocomposite scaffolds.

### 4.3 Characterizations

#### 4.3.1 Physicochemical analysis

The dried products before and after immersion in SBF were characterized by different techniques. The size of the particles was determined by transmission electron microscopy, (TEM, Hitachi H-7500 Japan) 120 kV. The hydrodynamic size of the particles was evaluated using dynamic light scattering measurements using Laser-Spectroscatter 201(RiNA) at 25 °C. Data was analyzed using PMgrv 3.01p17 software.
supplied with the instrument. SEM images at different magnifications were recorded using Scanning electron microscope JEOL-JAPAN, equipped with an energy dispersive X-Ray spectroscope EDX. The FTIR spectra of n-HA/CS-ST and n-HA/CS 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 recorded on Philips PW1710 diffractometer with Cu K\(\alpha\) radiation at 1.540 Å in the range of 20\(^{0}\)- 60\(^{0}\) at 40 kV. The thermal stability of the samples was investigated by thermogravimetric analysis (TGA) and differential thermal analysis (DTA) studies of the samples carried out on Shimadzu DTG-60H system (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 of the samples was determined using a 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 4505). Cylindrical specimens were prepared with dimensions 1 x 1 cm\(^2\). The testing conditions were at room temperature. The crosshead speed was set at 10 mm/min and the load was applied until the sample was fractured. The compressive strength was calculated from Equation 1:

\[ CS = 4P / \pi d^2 \]

(1)

where \(P\) is the load at the fracture point and \(d\) is the diameter (mm) of the cylindrical specimen. Three parallel samples were tested for every scaffold and the mean value of the compressive strength and shore hardness of different scaffolds were given.

### 4.3.2 Immersion in Simulated Body Fluid (SBF) study: \textit{in–vitro} analysis

The \textit{in-vitro} bioactivity of n-HA/CS-ST and n-HA/CS nanocomposites was investigated on the pellets (6mm in diameter and 2mm in thickness) made from dried samples of nanocomposites immersed into a tube containing 20 ml of SBF solution (having ion concentrations similar to human blood plasma) oscillating at 37.0 ± 0.5 °C in the water bath to allow the soaking of SBF solution [22,23]. The SBF solution was prepared by dissolving reagent chemicals of NaCl (7.996gm), NaHCO\(_3\) (0.350gm), KCl (0.224gm), K\(_2\)HPO\(_4\).3H\(_2\)O (0.228gm), MgCl\(_2\).6H\(_2\)O (0.305gm), CaCl\(_2\) (0.278gm) and Na\(_2\)SO\(_4\) (0.071gm) into 900ml distilled water. The fluid was buffered at physiological pH 7.40 at 37 °C with tri-(hydroxyl-methyl) amino methane [TRIS]
(6.057 gm) and HCl, and the solution was made up to 1000 ml with additional water. The pellets were withdrawn from SBF after soaking for 2, 4 and 8 weeks period and gently rinsed with double distilled water and dried.

4.3.3 Swelling test

To determine the percentage of water absorption, swelling studies were performed in simulated body fluid (SBF) at pH 7.4 and temperature of 37 °C. Nanocomposite scaffolds were made into small pellets and dry weights of the pellets ($W_d$) and the weights of soaked pellets for (1, 7, 14, 21 and 28 days) ($W_w$) in SBF solution at pH 7.4 were taken after removing the adsorbed water on the surface of the soaked pellets by filter paper. The swelling percentage was determined using Equation 2:

$$\% S = \frac{(W_w - W_d)}{W_d} \times 100$$ (2)

where $\% S$ is the swelling percentage. All samples were triplicated in the experiment.

4.3.4 Cytotoxicity assay

The cellular toxicity assay of nanocomposites, n-HA/CS-ST and n-HA/CS, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) was carried out by culturing murine fibroblast L929 cells (NCCS, Pune, India) in a contact mode as reported earlier [24]. In brief, L929 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and seeded on the films at their exponential phase of growth at a density of $10^5$ cells/cm$^2$. Various concentrations (from 0 to 50 mg/ml) of the nanocomposites n-HA/CS-ST and n-HA/CS were added to the monolayer. The cells were allowed to attach on the films surface for 3 hours in 5% CO$_2$ incubator at 37 °C. The fresh DMEM medium supplemented with 10% fetal calf serum (FCS-GIBCO, USA) was added to each well to keep the cell containing films submerged. The plates were incubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO$_2$ in air. The MTT (4mg/ml) was added to each well at strength of 10% (v/v) followed by incubation for another 4 hours at 37 °C. The media containing MTT was removed and 200 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured using an ELISA plate reader (Biorad, USA) at 595 nm. The resulting absorbance values recorded gave a quantitative measure of viable cells in terms of the cell population. The % proliferation was calculated and plot was generated using excel (Microsoft office-2007). The cytotoxicity of both the
nanocomposites was also evaluated on human osteoblasts-like MG-63 cells (NCCS Pune). The experiments were triplicated for both the nanocomposites samples. The experimental values were analyzed using Student’s t-test and p-value of <0.05 was considered statistically significant.

4.3.5 Bacterial Strains

A total of 35 isolates of Staphylococcus Aureus (Gram-positive bacteria) and Escherichia coli (Gram-negative bacteria) were obtained from the stocks culture, Department of Microbiology, Jawaharlal Nehru Medical College (JNMC), Aligarh Muslim University (AMU), Aligarh, India [25]. The isolates were originally isolated from the pus/wounds samples of the registered patients at JNMC. The identified and characterized isolates have been stored as glycerol cultures at -20 °C. The stock glycerol cultures of the clinical isolates were sub cultured in Luria Bertaini (LB) (Hi-Media, Mumbai, India) broth and maintained as pure cultures until used for testing [26]. S.Aureus ATCC 25923 and E.coli ATCC 25922 (Hi-Media, Mumbai, India) were used as standard control strains.

4.3.6 Assays for MIC and MBC of n-HA/CS and n-HA/CS-ST nanocomposites

Minimal Inhibitory Concentration (MIC)

S.Aureus and E.coli isolates were grown overnight on MHA plates at 35 °C before being used. The antibacterial activity of n-HA/CS and n-HA/CS-ST were examined using the standard agar dilution method [27]. The MIC was determined on MHA plates using serial two-fold dilutions of both the nanocomposites in concentration range from 3200 to 62 μg/ml. The initial bacterial inoculum of $2.5 \times 10^5$ CFU/ml, and the temperature and time of incubation at 37 °C and 24 hour, respectively were maintained throughout the experiments. The MIC is defined as the lowest concentration of the nanocomposites samples that resulted in no visible growth of the bacterial strains. The MIC measurements were done in triplicate to ascertain the value of MIC for each tested bacteria.

Minimal Bactericidal Concentration (MBC)

After the MIC determination of the nanocomposites an aliquots of 25μl from all tubes in which no visible bacterial growth was observed were seeded on to MHA plates not
supplemented with the nanocomposites and incubated for 24 hour at 37 °C. The MBC endpoint is defined as the lowest concentration of antibacterial agent that kills 100% of the initial bacterial population.

4.4 Results and Discussion

4.4.1 TEM and DLS studies

The TEM micrographs of n-HA/CS and n-HA/CS-ST nanocomposites [Figure 1] display that in n-HA/CS scaffold the particles were found to be severely agglomerated as compared to that seen in n-HA/CS-ST scaffold where particles were relatively in homogeneous dispersed state indicating that the presence of ST inhibited the aggregation of n-HA/CS particles in n-HA/CS-ST nanocomposite similar to that reported by Lei Yang et. al.[28]. The rod shaped particles of n-HA/CS-ST with an average size in the range of ~12-17 nm which were comparatively lower than the average size of n-HA/CS particles (~20-30 nm). These results were further confirmed by DLS studies [Figure 1(a)] and an average particle size in the range of ~15-30 nm and ~30-50 nm have been observed in the case of n-HA/CS-ST and n-HA/CS nanocomposites respectively complementing the TEM results. However, the slightly greater value of average sizes for both the nanocomposites compared to TEM arises from the fact that DLS calculates the hydrodynamic size of the particles involving the solvent layer at the interface. Therefore, these findings suggested that the incorporation of starch in n-HAP/CS matrix appears to control the size of the particles.

Figure 1: TEM micrographs of (a) n-HA/CS and (b) n-HA/CS-ST nanocomposites
Figure 1(a): DLS measurements of (a) n-HA/CS-ST (b) n-HA/CS nanocomposites

4.4.2 SEM

The comparison of the SEM micrographs of the n-HA/CS and n-HA/CS-ST nanocomposites depicted from Figure 2(a,b) revealed that n-HA/CS-ST has relatively rough and porous surface as compared to smoother and packed surface of n-HA/CS suggesting that the addition of ST influenced the surface morphology by modifying the n-HA/CS matrix, an important requirement favoring the tissue in-growth, bone formation and biological fixation with surrounding tissue [29]. The above discussion suggested that there is an ample possibility for interaction of OH\(^{-}\) of ST and NH\(_2\) of CS with each other and also with n-HA in n-HA/CS-ST scaffold leading to changes in physical properties of this scaffold relative to n-HA/CS scaffold [6]. This has been confirmed by comparing the mechanical properties of these two scaffolds where n-HA/CS-ST has shown greater hardness as compared to n-HA/CS.

4.4.3 In-vitro bioactivity evaluation of n-HA/CS-ST and n-HA/CS nanocomposite scaffolds

Generally, it is believed that the in-vitro calcification ability of biomaterials has a correlation with the bone-bonding ability in-vivo. Bioactivity is a result of the chemical reactions occurring at the surface of a material exposed to body fluids in order to the form a surface layer of hydroxyl carbonated apatite (HCA) upon
implantation which is an essential criterion for establishing bonding with natural bone. Thus, investigating the biological behavior of bioceramics in SBF is considered to be the most efficient method to authenticate their bioactivity in the body environment. *In-vitro* bioactivity of the nanocomposites n-HA/CS-ST and n-HA/CS scaffolds has been monitored by SEM microphotographs of the two scaffolds [Figure 2(c-h)] soaked in SBF solution with ionic concentration analogous to blood plasma at pH 7.40 and 36.5 °C for 2, 4 and 8 weeks. It has been observed that there was considerable biomimetic deposition of HA in both the matrix surfaces but n-HA/CS-ST matrix exhibited greater deposition in the form of thick layer as compared to n-HA/CS. The comparison of SEM images of both the scaffolds soaked in SBF for 8 weeks revealed that a thick apatite layer deposited throughout the n-HA/CS-ST nanocomposite as compared to n-HA/CS scaffold. In addition, a critical comparison of SEM micrographs of n-HA/CS and n-HA/CS-ST nanocomposites taken after time period of 8 weeks [Figure 2(g,h)] revealed curved rod like structures possibly of n-HA irregularly embedded in SEM micrograph of n-HA/CS-ST with length of about 20-25 nm in CS-ST matrix which could not be observed in n-HA/CS micrograph suggesting that the presence of ST induced higher growth of HA nanoparticles. These results suggested a promising potential bonding ability of n-HA/CS-ST nanocomposite that facilitates bone ingrowth formation and good osteointegration *in-vivo*.

4.4.4 Energy Dispersive X-ray Spectroscopy (EDX)

The comparative study of EDX spectra of n-HA/CS, n-HA/CS-ST and their respective SBF scaffolds kept for 2, 4 and 8 weeks shown in Figure 3(a-h) has been made. The observed semiquantitative ratio of Ca/P of 1.00 against the expected range of 1.67±0.67 in natural bone has been found in the EDX spectra of n-HA/CS-ST nanocomposite scaffolds kept in SBF for 2 weeks [Figure 3(a-d)] [30]. However n-HA/CS-ST scaffold kept for 8 weeks [Figure 3(g,h)] in SBF gave Ca/P value of 1.58 much closer to the theoretical stoichiometric ratio of hydroxyapatite (Ca/P=1.67) [31] as compared to the value of 1.84 obtained in case of n-HA/CS scaffold kept in SBF for 8 weeks that could be attributed to an appreciable increase in elemental concentrations of Ca and P (precursors for HA formation) along with other elements.
4.4.5 FTIR Analysis

The possible interaction between the various components in n-HA/CS-ST has been graphically displayed in Scheme 1. The preliminary information regarding the interaction of different phases in n-HA/CS and n-HA/CS-ST scaffolds has been obtained by comparing the FTIR spectra. The FTIR spectra of n-HA/CS, n-HA/CS-ST and n-HA/CS-ST-SBF (8 weeks) exhibit bands characteristic of n-HA, CS and ST moieties [Figure 4] in their respective scaffolds. In the FTIR spectrum of n-HA/CS, the presence of HA in CS matrix can be identified by its characteristic bands of phosphate group at 467, 563 and 602 cm\(^{-1}\) 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 discerns as a broad band in the region of 950 cm\(^{-1}\)-1034 cm\(^{-1}\)[32], while OH stretching band of HA gets overlapped with the OH stretching band of CS and a broad peak at about 3430 cm\(^{-1}\) appeared. The peaks at 1458 and 2927 cm\(^{-1}\) may reasonably be assigned to C-H stretching of chitosan [33], while bands at 1540 cm\(^{-1}\) and 1655 cm\(^{-1}\) represented N-H bending (amide II) and C=O stretching (amide I), respectively [34]. The FTIR spectrum of n-HA/CS-ST shows all characteristic bands corresponding to n-HA and CS at expected positions along with a wide band at 3436 cm\(^{-1}\) attributable to O-H stretching of amylopectin with its width warranting the formation of inter and intra-molecular hydrogen bonding. A slight modification in CS band appearance characteristic of aliphatic C-H stretching band at 2927 cm\(^{-1}\) may be due to C-H asymmetric stretching band of starch expected to appear in this range. The bands at 1432 and 1385 cm\(^{-1}\) may arise due to the angular deformation of C-H bonds in ST molecule. A positive shift in (amide II) in n-HA/CS from 1540 cm\(^{-1}\) to 1620 cm\(^{-1}\) in n-HA/CS-ST refers to the possible H-bonding between OH of starch and amino group of CS [34].
Figure 2: SEM micrographs of (a) n-HA/CS (b) n-HA/CS-ST and their respective SBF study after 2, 4 and 8 weeks (c-h).
Figure 3: EDX micrographs of (a) n-HA/CS (b) n-HA/CS-ST and their respective SBF study after 2, 4 and 8 weeks (c-h).
Moreover slight shifts in the phosphate group vibrations of HA in n-HA/CS-ST scaffold indicate that the presence of ST incited the dissociation and interaction of polymer with nucleating crystal [35]. The FTIR spectrum of n-HA/CS-ST kept in SBF for 8 weeks [Figure 4] exhibit the bands assignable to OH and H$_2$O along with a new weak intensity bands at 874 cm$^{-1}$ (v2), 1416 and 1462 cm$^{-1}$ (v3) characteristic of CO$_3^{2-}$ indicative of the formation of small amount of CO$_3^{2-}$ moiety in n-HA in presence of ST [6] which is advantageous for bone mineral (expected to be 4-8 wt% in the human body), as it enhance the mechanical consistency and bioactivity of the apatite leading to more osteoconduction and tissue in-growth is expected on implantation. A slight broadening in band at 1037 cm$^{-1}$ and small shifts in PO$_4^{3-}$ stretching modes may arise in view of the distortion of HA crystalline structure due to replacing phosphate groups by carbonate groups [36].

Scheme 1: Possible interaction between different components in n-HA/CS-ST nanocomposite.
4.4.6 X-ray diffraction (XRD) studies

The X-ray diffraction patterns of the nanocomposites n-HA/CS (a), n-HA/CS-ST (b), n-HA/CS-ST-SBF (8 weeks) (c) and original human bone (d) are shown in Figure 5(a-d). The average crystallite sizes of nanocomposites and the human bone were calculated using Scherrer’s equation:

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

where \( L \) is the average crystallite size, \( \beta \) is the full width of the peak at half of maximum intensity (rad) (FWHM) [1,37], \( \lambda \) is the wavelength of monochromatic X-ray beam radiation Cu radiation (\( \lambda = 1.5406 \, \text{Å} \)), \( \theta \) 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 appears at \( 2\theta = 26, 29.3, 32.3 \) etc. which confirms the presence of n-HA crystallites. In Figure 5(a-c), the presence of these characteristic peaks confirmed the presence of n-HA in all the scaffolds which matches well with the XRD peaks of original human bone displayed in Figure 5d, indicating that crystallization of n-HA still existed after nanocomposite formation which could be resulted from interface binding between n-HA particles and polymers matrix [38]. The average crystallite size of all the four scaffolds were
calculated by Scherer equation confirming the nanostructure of the nanocomposites and were found to be 24.1 nm for n-HA/CS-ST (SBF-8weeks) and 20.0 nm for n-HA/CS as compared to 12.9 nm for n-HA/CS-ST and 14.7 nm for human bone. The increase in average crystallite size of n-HA/CS-ST immersed in SBF solution for 8 weeks may be explained in terms of growth in number and size of n-HA particles leading to complete coverage of polymer matrix by apatite layer [29]. Thus the immersion of n-HA/CS-ST for sufficient time period in SBF enhances ability of formation and nucleation of apatite layer which is triggered by the addition of ST possibly due to greater interaction in n-HA/CS-ST scaffold.

Figure 5: X-ray diffractograms of (a) n-HA/CS (b) n-HA/CS-ST (c) n-HA/CS-ST-SBF (8 weeks) and (d) Bone

4.4.7 TGA-DTA Analysis:

In order to meet out the biocompatibility of the biomaterials, it is important to verify the thermal stability via TGA analysis not only in the temperature range of human body but also in higher temperature intervals which involves sterilization processes. The TGA and DTA curves of CS, n-HA/CS, n-HA/CS-ST and n-HA/CS-ST-SBF-8 weeks have been displayed in Figure 6 and Figure 7, respectively. The TGA curve of CS represented the two step weight loss in the range of 90-130 °C and 240-400 °C corresponding to loss of adsorbed water molecule present in the scaffold and the decomposition of CS, respectively as depicted from Figure 6. The presence of one endothermic and one exothermic peak in DTA curve of CS in Figure 7 further support TGA curve. However the TGA graph of n-HA/CS nanocomposite comprises of two
step weight loss in the range of 85-150 °C and 225 - 410 °C in consistency with DTA curve, having total weight loss of about (72-75) % compared to (85-90) % in case of CS suggesting that the n-HA enhanced the thermal stability of CS. Further, the thermal stability of n-HA/CS scaffold got increased by incorporation of ST in n-HA/CS-ST which shows two step weight loss in the range of 80-140 °C and 260- 380 °C corresponds to moisture loss and decomposition of organic moieties, respectively which is in agreement with its DTA curve, resulting in decrease in total weight loss in the range of (35-40) % with the incorporation of starch. The TGA curve of n-HA/CS-ST (SBF-8 weeks) represented that the initial degradation temperature was shifted slightly to lower temperature which indicates lesser total weight loss of about (25-30) % in the temperature range of 65-300 °C attaining stability beyond 300 °C suggesting that the total weight loss due to the thermal decomposition of the nanocomposite (n-HA/CS-ST-8 weeks) decreased, as the inorganic content (HA) in the nanocomposite increased. The higher stability of n-HA/CS-ST (SBF-8weeks) comparative to other scaffolds may be explained in terms of increase amount of hydroxyapatite in the polymer matrix in presence of ST after immersing in SBF solution for 8 weeks which strongly supports superior in-vitro bioactivity of n-HA/CS-ST (soaked in SBF for 2,4 and 8 weeks) nanocomposite compared to n-HA/CS corroborated from SEM studies [29,39]. Thus comparative study of thermal analysis of the various scaffolds suggested that ST significantly raised the thermal stability of n-HA/CS nanocomposite in n-HA/CS-ST [40], possibly due to regular increased interactions.

![TGA curves of CS, n-HA/CS, n-HA/CS-ST and n-HA/CS-ST(SBF-8weeks) scaffolds.](image)

Figure 6: TGA curves of CS, n-HA/CS, n-HA/CS-ST and n-HA/CS-ST(SBF-8weeks) scaffolds.
4.4.8 Swelling Test

The study of swelling percentage of n-HA/CS and n-HA/CS-ST nanocomposites in SBF solution for different time intervals (1,7,14,21 and 28 days) displayed in Figure 8, revealed a regular decrease in swelling capacity of both the scaffolds on increase in time intervals. However, a comparative analysis of swelling percentages of n-HA/CS and n-HA/CS-ST indicate that n-HA/CS-ST has lower swelling capacity as compared to n-HA/CS [Figure 8] on increase in time intervals which may be explained in terms of increased interactions between n-HA,CS and ST as compared to n-HA/CS [41,42].

![Swelling Test Graph](image)

**Figure 7:** DTA curves of CS, n-HA/CS, n-HA/CS-ST and n-HA/CS-ST (SBF-8 weeks) scaffolds.

![Swelling Test Graph](image)

**Figure 8:** Swelling percentage of n-HA/CS and n-HA/CS-ST nanocomposites.
4.4.9 In-vitro toxicity of nanocomposite

The nanocomposites scaffolds n-HA/CS-ST and n-HA/CS were subjected to cytotoxic studies with murine fibroblast L929 and human osteoblasts-like MG-63 cells. The cells were incubated with various concentrations (0-50 mg/ml). The cellular toxicity of these nanocomposites was evaluated using MTT assay after 24 hours. MTT assay revealed significant non-toxic nature of n-HA/CS-ST to both the cell lines even at higher concentrations (25-50 mg/ml) as compared to n-HA/CS at similar concentrations [Figure 9a and 9b]. In other words n-HA/CS-ST showed significant superior biocompatibility without interfering the cellular machinery over n-HA/CS confirming excellent in-vitro cytocompatibility. These comparative results suggest that n-HA/CS-ST would be a promising candidate for bone tissue engineering in search of a scaffold to be used as bone implant for orthopaedic applications in mammals [43].

**Figure 9:** Cellular toxicity of n-HA/CS and n-HA/CS-ST nanocomposites determined by MTT assay on: (a) L929 cells (b) MG-63 cells.

4.4.10 MIC and MBC of n-HA/CS and n-HA/CS-ST

The antibacterial activity results suggested that both the scaffolds exhibited antibacterial properties for both Gram positive and Gram-negative bacteria. However it is found that MIC and MBC values for n-HA/CS-ST were lower in comparison to that shown by n-HA/CS [Table 1]. The superior antibacterial nature of n-HA/CS-ST as compared to n-HA/CS may be due to relatively smaller particle size of n-HA/CS-ST [1] enhancing surface area to volume ratio leading to higher antibacterial activity.
The greater antibacterial activity of n-HA/CS-ST may also be explained by relating higher interaction ability of small sized particles of n-HA/CS-ST with bacteria resulting higher disruption of cell membranes and destruction of cytoplasm [44].

Table 1: MIC and MBC of n-HA/CS-ST and n-HA/CS nanocomposites.

<table>
<thead>
<tr>
<th>Nanocposite</th>
<th>Bacterial Strains</th>
<th>MIC(µg/ml)</th>
<th>MBC(µg/ml)</th>
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</thead>
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<tr>
<td>n-HA/CS-ST</td>
<td>S.Aureus</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>E.Coli</td>
<td>1000</td>
<td>2000</td>
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4.4.11 Mechanical Properties

4.4.11.1 Shore hardness and compressive strength of nanocomposites

The initial mechanical properties are usually important criteria in choosing the scaffold materials for bone tissue engineering. Shore hardness measured by a scleroscopic hardness tester is a kind of dynamic hardness which measures the height of the bounce of a diamond tipped hammer dropped from a fixed height on the sample to be analyzed [45]. A comparative study of shore hardness of CS, n-HA/CS and n-HA/CS-ST performed on their pellets shown in Figure 10(a-c), revealed the average value of shore hardness of 33 ±2.88, 45 ±7.63 and 69 ±5.29 respectively [Figure 10(e)]. The highest value of shore hardness in n-HA/CS-ST nanocomposite relative to CS, n-HA/CS may be explained in terms of possible highest interaction display between n-HA/CS and ST. The compressive strength is another mechanical property to be considered in orthopedics particularly for replacement of cancellous bone [46]. A comparative study of compressive strengths of CS, n-HA/CS and n-HA/CS-ST revealed average compressive strengths of (3.00±0.09 MPa), (5.0±0.15 MPa) and (9±0.165 MPa), respectively [Figure 10(d)]. The compressive strength of n-HA/CS-ST seems to be most satisfactory when compared to the natural bone (2-10MPa) [22, 47-48]. Thus it may be concluded that n-HA/CS-ST nanocomposite could be a promising candidate for new bone tissue regeneration at the site of implantation maintaining sufficient integrity.
4.5 Conclusion

A potentially bioactive n-HA/CS-ST nanocomposite was synthesized by co-precipitation method at room temperature to investigate its viability for bone tissue engineering applications. The FTIR spectra of n-HA/CS and n-HA/CS-ST scaffolds exhibited the bands characteristic of organic and inorganic moieties indicating significant intermolecular interaction between the different components in both the nanocomposites. The comparative XRD results of the two scaffolds revealed that the size of n-HA/CS decreased upon incorporation of ST. The increase in thermal stability in n-HA/CS-ST nanocomposite as compared to n-HA/CS has been observed by comparing the TGA results. The comparison of SEM images of both the scaffolds indicated that the addition of ST influenced the surface morphology of n-HA/CS.
scaffold which appeared to be more rough and porous. A considerable improvement in the values of shore hardness and compressive strength of the n-HA/CS-ST scaffold as compared to CS and n-HA/CS indicated relatively increased interactions. The comparison of SBF studies monitored by SEM images of n-HA/CS and n-HA/CS-ST scaffolds suggested a better bioactivity of n-HA/CS-ST indicating the greater ability to facilitate bone ingrowth formation and possibility of good osteointegration \textit{in-vivo}. The MTT assay studies on n-HA/CS and n-HA/CS-ST revealed higher cell proliferation in n-HA/CS-ST compared to n-HA/CS warranting a superior non-toxicity. The comparison of results of antibacterial property of n-HA/CS-ST and n-HA/CS against both Gram-positive and Gram-negative bacteria revealed an improved antibacterial property of n-HA/CS-ST. Thus, these findings on n-HA/CS-ST nanocomposite would be a step forward in the development of a competent bone construct in the field of Bone tissue engineering.
Chapter 4  Nano-Hydroxyapatite/Chitosan-Starch Nanocomposite

References


