Chapter 3

Materials and methods

This chapter describes the synthesis and characterization of various material Hydroxyapatite (HA), Sodium Potassium Niobate (NKN), 45S5 Bioglass (BG), 1393 Bioglass (BG), as well as (1-x) HA – x NKN, (1-x) 45S5 BG – x NKN and (1-x)1393 BG – x NKN composites. Phase evolution (XRD and FTIR), microstructural characterization (SEM) are demonstrated. Mechanical properties (hardness, fracture toughness, compressive strength and flexural strength), dielectric and electrical behavior (dielectric constant, loss, AC conductivity and impedance analyses), antibacterial response (quantitatively and qualitatively) and cellular response with combined effect of surface charge and electric stimulation are also discussed.

3.1 Synthesis of Hydroxyapatite (HA)

The synthesis of HA has been done using co-precipitation method. CaO (Merck life science Pvt. Ltd., 90 %) and H_3PO_4 (Loba Chemie, 85 %). were used as raw material. Fig. 3.1 represents the synthesis flow chart for HA. Initially, CaO powder was weighed by weighing balance (Shimadzu) and kept it in oven at 100ºC for 6-8 h to remove the moisture of the powder. Dried powder was mixed with double ionized (DI) water. For 25 g batch, CaO and DI water have been taken to be 15.49 g and 750 ml, respectively. The mixing was done by magnetic stirrer at temperature of 80ºC. 9.5 ml of orthophosphoric acid (H_3PO_4) was diluted with 1000 ml of DI water. The diluted H_3PO_4 solution was poured in to funnel and arrange or stand over CaO solution beaker. Then, mix diluted H_3PO_4 solution was added drop wise in to CaO solution beaker. The entire mixing procedure takes about 3 – 4 h. The pH of the solution was maintained (> 8) throughout the mixing process. After the completion of mixing, the solution was kept for 1 day for

precipitation. The precipitate was collected by filtration. The filtered precipitate was kept overnight at 100ºC in oven for drying.

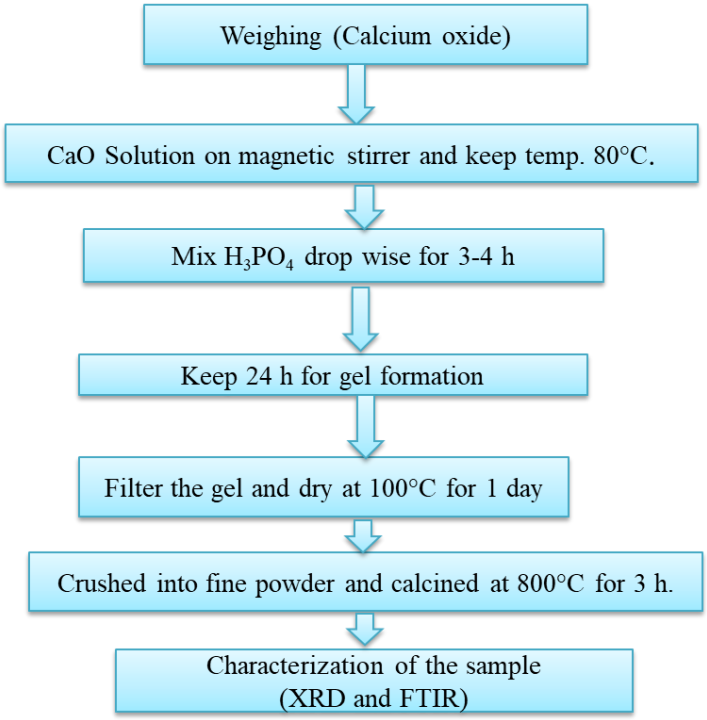

Fig. 3.1 Flow chart representing the synthesis of Hydroxyapatite (HA)

Now, the dried powder was crushed into fine particle and calcined at 800ºC for 2 h. Eq. 3.1 shows the formation of HA after calcinations.

$$\mathbf{10\ CaO\ + 6\ H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2\ + 18\ H_2O} \qquad \mathbf{(3.1)}$$

The calcined powder was crushed further and characterized to confirm the formation of pure phase of HA.

3.2 Synthesis of Sodium Potassium Niobate ($Na_{0.5}K_{0.5}NbO_3$, NKN)

The synthesis of NKN was done using solid state synthesis method. Na_2CO_3 (Sigma Aldrich, 99 %), K_2CO_3 (Sigma Aldrich, 99%) and Nb_2O_5 (Sigma Aldrich, 99.5 %) were used as raw material for synthesis of NKN.

The precursors were mixed via ball milling using zirconia balls (1:4 powder to ball ratio) with acetone media in polyethylene jar for 24 h. Wet milled slurry was then poured in beaker and kept overnight in oven at 100ºC for drying. The dried powder was crushed using agate mortar pestle to make powder fine. Then, the powder was calcined at 910ºC for 2 h. Eq. 3.2 shows the formation of HA after calcinations.

$$\frac{1}{4}\,Na_2CO_3 + \frac{1}{4}\,K_2CO_3 + \frac{1}{2}\,Nb_2O_5 \rightarrow Na_{0.5}K_{0.5}NbO_3 + \frac{1}{2}\,CO_2 \qquad (3.2)$$

Calcined powder was crushed further in to fine particles and characterizations were done to assure the formation of pure phase of NKN.

3.3 Synthesis of HA – NKN composites

(1-x) HA – x NKN (x= 10, 20 and 30 wt. %) composite systems were fabricated via solid state synthesis route. The mixing of HA and NKN samples was done by ball milling using zirconia balls (1:4 powder to ball ratio) and acetone (as milling media) in polyethylene jar for 24 h. The wet slurry of HA and NKN was kept overnight in oven for drying. The dried cake was crushed into fine particles. Table no. 3.1 shows the composition and nomenclature for (1-x) HA-x NKN which have been used throughout the thesis.

Table 3.1 Composition and nomenclature used for HA –NKN composites

S. No.	HA (wt.)%	NKN (wt. %)	Nomenclature
1.	90	10	HA – 10 NKN
2.	80	20	HA – 20 NKN
3.	70	30	HA – 30 NKN

3.4 Synthesis of 45S5 Bioglass (45S5 BG)

45S5 BG was synthesized using melt quenching method using raw material SiO_2 (Loba Chemie), Na_2CO_3 (Sigma Aldrich, 99%), $(NH_4)_2HPO_4$ (Loba Chemie, 99%) and $CaCO_3$

(Loba Chemie, 98%). The flow chart for synthesis of 45S5 BG is shown as Fig. 3.2.

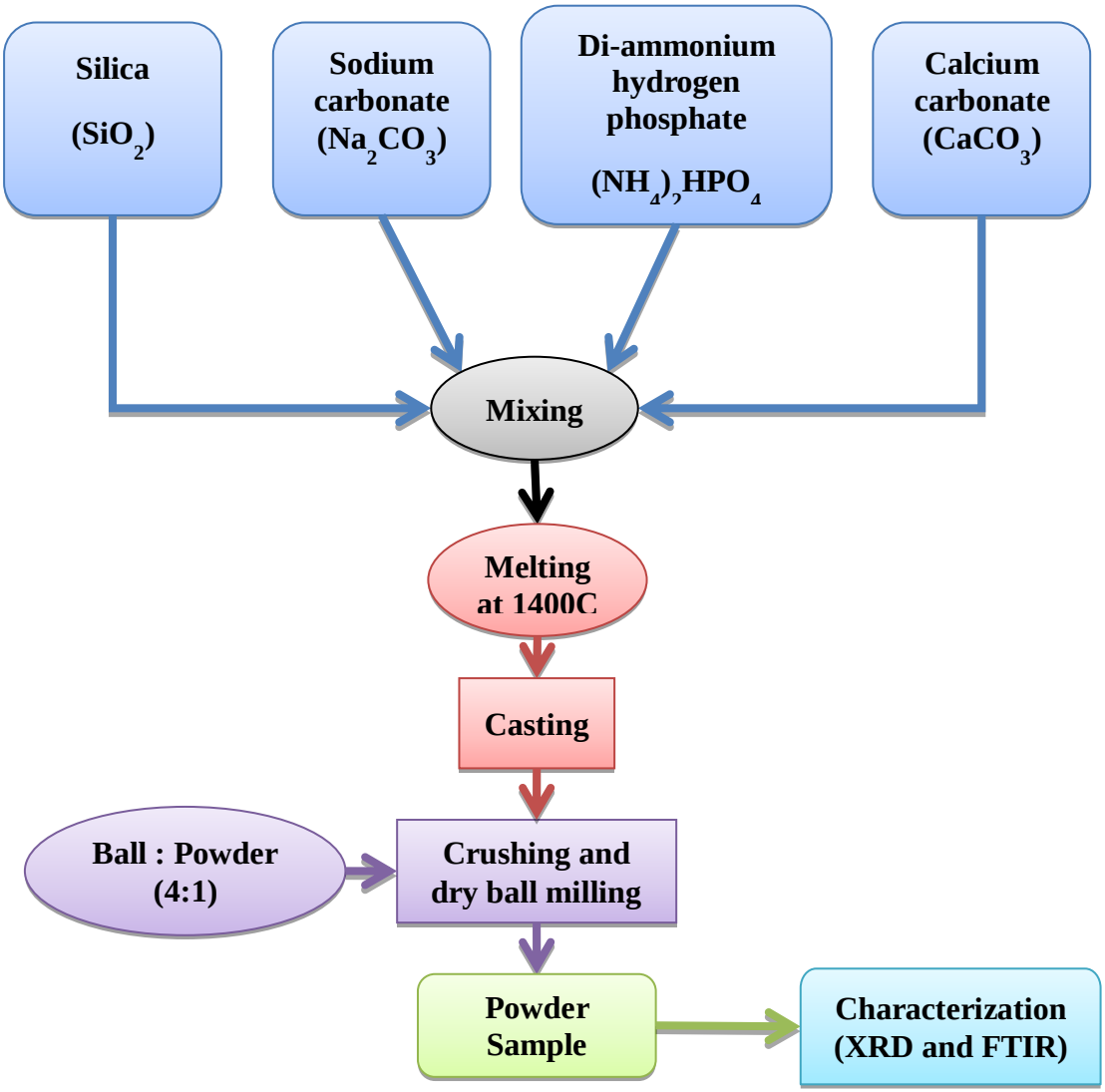

Fig. 3.2 Flow chart for the synthesis of 45S5 bioglass (BG)

Table 3.2 Composition of 45S5 BG

Composition	SiO_2	Na_2O	CaO	P_2O_5
Weight %	45	24.5	24.5	6

The raw materials (as per table 3.2) were weighed using and mix to them in agate mortar pestle. The mixed powders were kept in furnace at 1400ºC for 2 h and melted in alumina crucible. Molten sample was then quenched in water and kept overnight in oven at 100°C

for drying. Dried glassy sample was dry ball milled to get the fine and uniform particles which were further characterized to assure the formation of 45S5 BG.

3.5 Synthesis of 45S5 BG - NKN composites

(1-x) 45S5 BG-x NKN composite was fabricated using solid state synthesis route. The mixing of BG and NKN samples was performed by ball milling using zirconia balls (1:4 powders to ball ratio) and ethanol (as milling media) in polyethylene jar for 24 h. The wet slurry of BG and NKN was kept overnight in oven for drying. The dried cake was crushed into fine particles. Table no. 3.3 shows the composition and nomenclature for (1-x) 45S5 BG – x NKN which have been used throughout the thesis.

Table 3.3 Composition and nomenclature used for (1-x) 45S5 BG – x NKN composites

S. No.	45S5 BG (vol.)%	NKN (vol. %)	Nomenclature
1.	90	10	BG – 10 NKN
2.	80	20	BG – 20 NKN
3.	70	30	BG – 30 NKN

3.6 Synthesis of 1393 bioglass (1393 BG)

45S5 BG was synthesized using melt quenching method, using raw material SiO_2, Na_2CO_3, $(NH_4)_2HPO_4$, K_2CO_3, $MgCO_3$ and $CaCO_3$. The flow chart for synthesis of 1393 BG is shown as Fig 3.3.

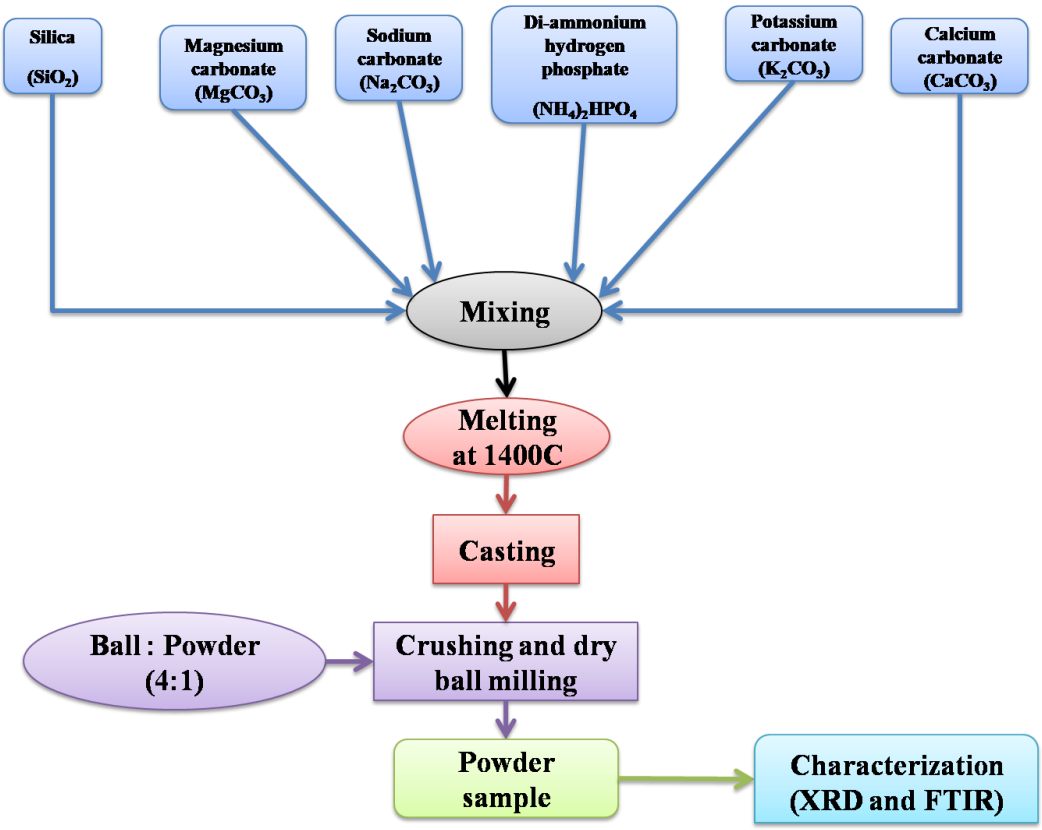

Fig. 3.3 Flow chart for the synthesis of 1393 bioglass (BG)

Table 3.4 Composition of 1393 BG

Composition	SiO_2	Na_2O	K_2O	MgO	CaO	P_2O_5
Weight %	53	6	12	5	20	4

The raw materials (Table 3.4) were weighed and mix in agate mortar pestle. The mixed powders were kept in furnace at 1400ºC for 4 h and melted in alumina crucible. Molten sample was then quenched in water and kept overnight in oven at 100ºC for drying. Dried glassy sample was dry ball milled to get the fine and uniform particles which were characterized to assure the formation of 1393 BG.

3.7 Synthesis of 1393 BG - NKN composites

(1-x) 1393 BG-x NKN composite was fabricated using solid state synthesis route. 1393 BG and NKN samples were mixed by ball milling using zirconia balls (1:4 powders to ball ratio) and ethanol (as milling media) in polyethylene jar for 24 h. The wet slurry of BG and NKN was kept overnight in oven for drying. The dried cake was crushed into fine particles. Table no. 3.5 shows the composition and nomenclature for (1-x) 1393 BG-x NKN which have been used throughout the thesis.

Table 3.5 Composition and nomenclature used for (1-x) 1393 BG – x NKN composites

S. No.	1393 BG (vol.)%	NKN (vol. %)	Nomenclature
1.	90	10	1393 BG – 10 NKN
2.	80	20	1393 BG – 20 NKN
3.	70	30	1393 BG – 30 NKN

3.8 Pelletization and sintering of composite samples

The pelletization of the powder samples was done by uniaxial hydraulic pressing at 5 MPa followed by cold isostatic pressing at 300 MPa [Fig. 3.4].

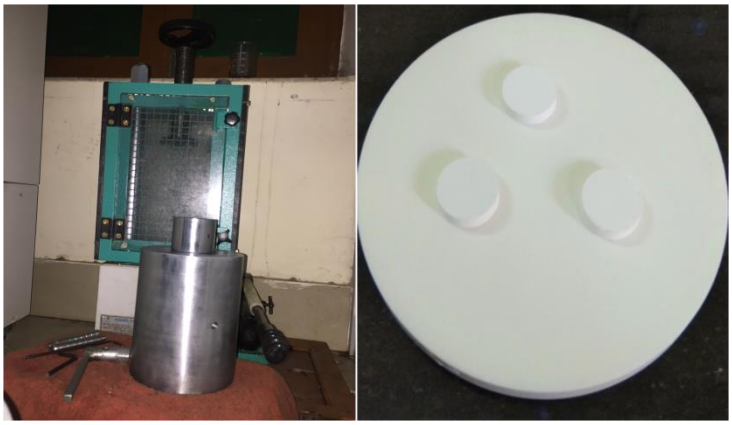

Fig. 3.4 Digital pictographs of compact samples using cold isostatic pressing (CIP)

The sintering temperature was optimized, based on the maximum densification as well as phase ratio. During optimization process, the samples were heated at heating rate of 5° / min Table 3.6 summarize the optimal sintering parameter for (1 – x) HA – x NKN, (1 – x) 45S5 BG – x NKN and (1 – x) 1393 BG – x NKN composites.

Table 3.6 Optimized sintering temperature for various composites

Samples	Optimized sintering temperature	Holding time
(1-x) HA- x NKN	1075°C	2 h
(1-x) 45S5 BG- xNKN	800°C	30 min
(1-x) 1393 BG- x NKN	800°C	30 min

3.8 Density measurement

Densities of the sintered samples were measured by Archimedes principal.

$$\textbf{Density of the bulk sample } (\rho_{actual}) = \frac{\text{Dry weight}}{\text{Weight loss}} = \frac{W_1}{W_1 - W_2}$$

Where, Dry weight of the sample (in air) = W_1

Suspended weight (in water) = W_2

Theoretical density

The theoretical densities for the composite samples were calculated using rule of mixture.

$$\textbf{Theoretical density } (\rho_{th}) = \frac{\rho_{matrix} V_{matrix} + \rho_{secondary\ phase} V_{secondary\ phase}}{V_{matrix} + V_{secondary\ phase}}$$

Thus, densification can be obtained by

$$\textbf{Densification } (\%) = \frac{\rho_{actual}}{\rho_{theoretical}} \times 100$$

3.9 Phase evolution

3.9.1 X-Ray diffraction

X-ray diffraction (XRD) patterns of the sintered samples were recorded using X-ray diffractometer (XRD, Rigaku Miniflex II Desktop X-ray Diffractometer) with Cu-Kα radiation (λ = 1.54056 Å). X-ray intensity was measured for angles in the range $20^{o} \leq 2\theta \leq 80^{o}$ with a step size of 0.05° and scan rate of 3° per minute. For identification of the phases, present in the sample, the diffraction patterns were then compared with the standard data in the JCPDS database and X pert high score software.

3.9.2 FTIR spectroscopy

The Fourier transformation infrared spectroscopy (FTIR) measurement was carried out in order to analyse the presence of functional groups. The IR spectra were measured in transmission mode within the range of 4000 - 400 cm^{-1} with a FTIR spectrometer (Bruker Tensor 70).

3.10 Scanning electron microscopy (SEM)

Microstructural analyses were done using scanning electron microscope (SEM, Zeiss, EVO 18 Research). It uses a focused beam of high-energy electrons in producing a variety of signals at the surface of specimen. The interacting electrons produced the signals which provide the information about the surface morphology etc. when the electron beam hits the surface, it may be either absorbed or reflected. The images were taken with different resolutions ranges from 500 x to 100000 x.

3.11 Mechanical characterization

3.11.1 Vicker's hardness measurement

Hardness is the resistance of the material to plastic deformation and meausred by standard indentation hardness test [1] The hardness of samples with 10 mm of diameter and 1.5 mm of thickness was measured by Vicker's hardness tester. This method consists of indenting the test material with a with pyramidal shaped diamond indenter. The indents were taken on the well-polished surfaces of the pellets with Vicker's diamond pyramid indenter (Digi-test, VTP-6046) at room temperature with 1 kgf of load with 10 s dwell time [Fig. 3.5].

The polishing process included grinding of the pellets on grinding papers with grades, 400-800,600-1200 and 2000. Finally, the samples were mirror polished using diamond polishing unit. Number of indents was taken on each sample. Vickers hardness (HV) was determined according to the method, described as ASTM E384 [2] and calculated by the formula (eq. 3.3),

$$HV = 1.854 \ {}^{F}/_{d^2} \qquad (3.3)$$

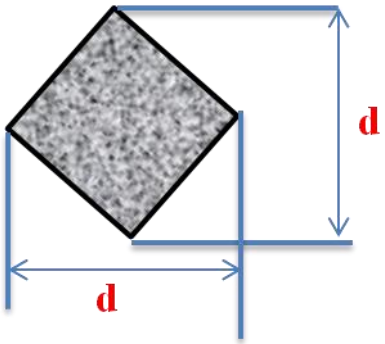

Fig. 3.5 Schematic diagram for indentation on the specimen at 1 kgf load with 10 s dwell time

Where, F is the load or force, applied to the diamond indenter in kilograms-force (kgf), d is the average length of the two diagonals.

3.11.2 Fracture toughness

Indentation method was used for calculating the fracture toughness of the disc samples with dimension of 10 mm × 1.5 mm. In this technique, crack lengths and crack radius of multiple indents were observed using SEM. Fig. 3.6 shows the schematic diagram for indentation by pyramidal indenter at 5 kgf load and the dwell time was 10 s.

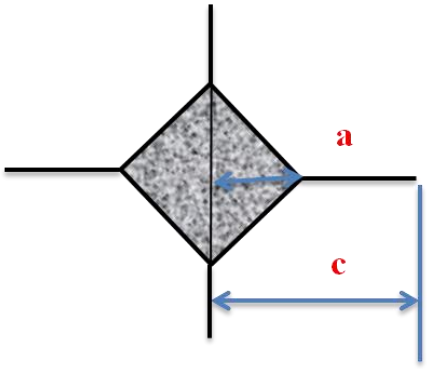

Fig. 3.6 Schematic diagram for crack generation on the specimen at 5 kgf load for 10 s dwell time

The fracture toughness (K_C) is evaluated by using Evans and Charles formula (eq. 3.4),[3]

$$K_C = \frac{0.15\, k\, H}{\Phi\sqrt{a}} (C)^{-3/2} \qquad (3.4)$$

Where,

k is correction factor (i.e, k = 3.2 for C/a values ≥ 2), H, C, ϕ and a are the hardness, crack length, constraint factor (ϕ = 3), and indent radius, respevtively.

3.11.3 Flexural strength

Flexural strength was measured using three point bend test method by universal testing machine (UTM, TInius Olsen). The rectangular bar shaped sample of dimension of 30 mm × 5 mm × 5 mm with crosshead speed of 0.05 mm/min were fractured [Fig. 3.7]. The flexural stress was calculated using the formula (eq. 3.5): [4]

$$\sigma_f = \frac{3PL}{2bd^2} \qquad (3.5)$$

Where,

σ_f is flexural strength, P is fracture load, L, b and d are span length, breadth and thickness of the specimen, respectively.

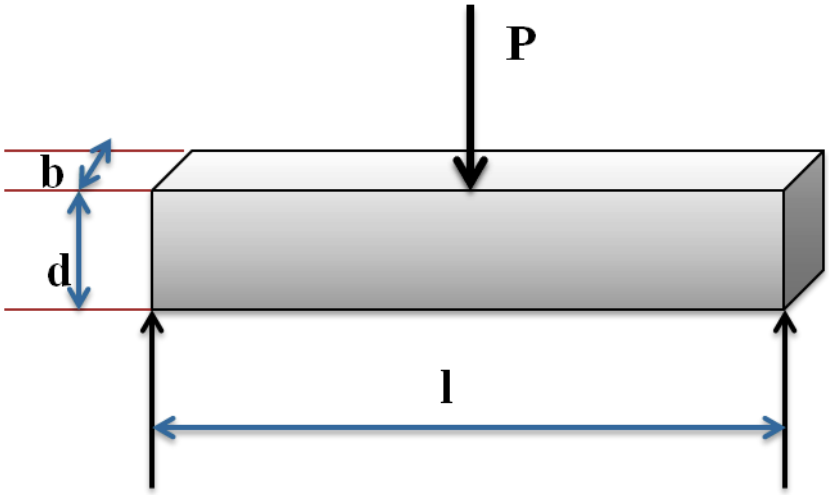

Fig. 3.7 Schematic diagram for rectangular specimen for three point flexural test

3.11.4 Compressive strength

The compressive strength of BG and BG – (10 - 30) NKN composite samples was measured using Universal testing machine (UTM, Tinius Olsen H10KL) at cross head speed of 0.05 mm/min. The diameter and thickness of the cylindrical samples was measured to be 10 mm and 10 mm, respectively [Fig. 3.8].

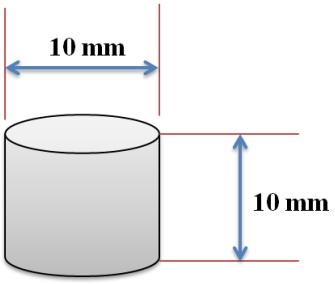

Fig. 3.8 Schematic diagram for cylindrical specimen for compressive strength measurement

3.12 Dielectric and electrical measurement

Dielectric and electrical properties such as dielectric constant, loss tangent, ac conductivity, impedance and modulus were measured in the temperature and frequency range of up to 500^{o}C and 1 MHz (from 1 Hz), respectively, using Nova control Alpha high performance frequency analyzer. For the electrical and dielectric measurements, the cylindrical samples with dimensions 10 mm × 1.5 mm were electroded with Ag paste and then cured at 600^{o}C for 5 min. The dielectric constant (eq. 3.6) and AC conductivity (3.7) were calculated using the formula,

$$\text{Dielectric constant, } \boldsymbol{\varepsilon_r = \frac{C \times d}{\varepsilon_0 A}} \qquad \textbf{(3.6)}$$

Where, ε_0 (8.854×10^{-12}) is the dielectric constant. C, d and A are the capacitance, thickness and area of the disc samples.

$$\text{AC conductivity, } \boldsymbol{\sigma_{ac} = \frac{G \times d}{A}} \qquad \textbf{(3.7)}$$

Where, G is the conductance (= ω × C × D), D is the dissipation factor, d and A are the thickness and area of disc samples, respectively.

3.13 Polarization treatment

The poling of the samples was done to observe the effect of surface charges induced by polarization on antibacterial as well as the cellular response of the samples. In this work, HA / 45S5 BG / 1393 BG - (10 - 30) NKN and monolithic HA / 45S5 BG / 1393 BG disc samples with 10 mm diameter and 1 mm thickness were polarized for 500ºC of temperatures at 20 kV for 30 min. Consequently, corona exposed surface has been negatively charged and induces the positive charge on opposite surface [Fig. 3.9].

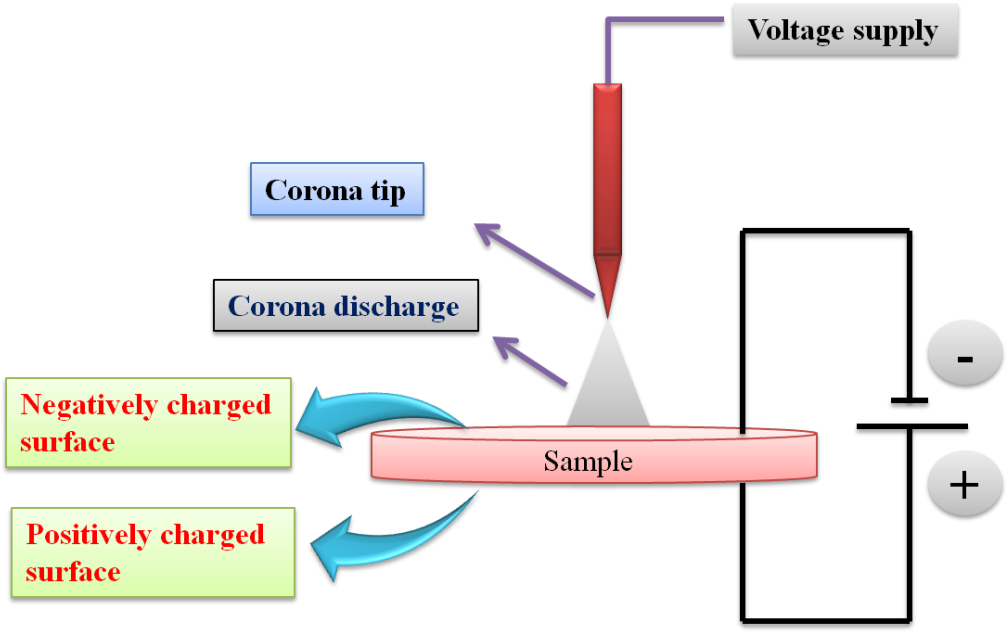

Fig 3.9 Schematic diagram for polarization of disc shaped samples at 20 kV for 30 min using corona poling unit

3.14 Polarization induced antibacterial behaviour

3.14.1 Quantitative analysis

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay of unpolarized and polarized HA / 45S5 BG / 1393 BG –NKN composite samples and monolithic HA / 45S5 BG / 1393 BG samples was performed to observe the viability of the bacterial cells in terms of optical density against the gram positive (Staphylococcus aureus; MTCC 435) and gram negative bacteria (Escherichia coli; MTCC 443). Both, the bacterial cells were revived in Nutrient Agar growth media for 10 h at 37ºC, before seeding on the samples. After washing with phosphate buffer saline (1x PBS), HA / 45S5 BG / 1393 BG –NKN and monolithic HA / 45S5 BG / 1393 BG samples were seeded with bacterial cells of 0.1 OD (200 µl per well) in 24 well plate and incubated for 6 h at 37ºC. The incubated samples were cleansed twice with 1x PBS and 500 µl of MTT was added in the well (MTT: PBS in the ratio of 1:10) and incubated further for 2 h to form

farmazan crystals which were dissolved with dimethyl sulfoxide (DMSO) [5]. The optical density of the solution was measured by ELISA Micro plate reader at wavelength of 595 nm and statistical analyses were done by SPSS 20 software. For statistical analysis of measured mean optical density, ANOVA method using Tukey test has been adopted at statistical significant value, $p < 0.05$.

3.14.2 Live / dead assay

Live / dead assay of S aureus and E. coli bacterial cells on HA / 1393 BG –NKN and monolithic HA / 1393 BG samples was performed to observed the viability of bacterial cells after incorporation piezoelectric NKN in monolithic HA / 1393 BG and subsequent polarization. Unpolarized and polarized HA / 1393 BG – NKN and monolithic HA / 1393 BG samples were seeded with bacterial (E. coli and S. aureus) cells (200 µl per well with 0.1 OD) in 24 well plate and incubated at 37ºC for 7 – 8 h. All the cultured samples were washed twice with 1x PBS solution after stipulated incubation period. Following this, a mixture of Syto 9 and propidium iodide dyes with the ratio of 9:1 was added for 30-40 min to stain the live and dead bacterial cells on the samples. After that, the imaging of live and dead bacterial cells on the unpolarized and polarized samples were performed using fluorescence microscopy

3.14.3 Nitro blue tetrazolium (NBT) assay

NBT assay was performed to evaluate the superoxide production (O_2^-) which is a type of reactive oxygen species (ROS). Both the S. aureus and E. coli bacterial cells were seeded on pre-sterilized polarized and unpolarized monolithic HA / 45S5 BG / 1393 BG and HA / 45S5 BG / 1393 BG – NKN composite samples and then incubated for 6 h under standard condition. After that 500 µl of NBT (10mg/ml in DI water) was added on the cultured samples and incubated further for 1 h. In presence of ROS, the yellow color NBT reduced to blue black colored formazan, which was dissolved in DMSO after stipulated

time period and optical density were measured using microplate reader (Biorad iMark^{TM}) at 595 nm.

3.15 Cell culture experiment

To evaluate the in vitro cytocompatibility of monolithic HA / 45S5 BG / 1393 BG and HA / 45S5 BG / 1393 BG – 30 NKN composite samples, osteoblast cells (MG-63, NCCS, Pune) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15 % fetal bovine serum (FBS) and 1 % antibiotics, and incubated in a humidified CO_2 (5 %) incubator (Thermo scientific Heracellvios 160i CO_2 incubator) at 37ºC. Before seeding, the samples as well as control disk were autoclaved at 121°C and 15 pound pressure for 15 min, except for the polarized samples. Following this, the samples were soaked in 70% ethanol along with UV exposure for 1 h, followed by washing with 1X PBS. After sterilization, the cells were trypsinised and an equal amount of cells (10^4 cells/ml) were seeded on glass disc (control), HA / 45S5 BG / 1393 BG [unpolarized (HA / 45S5 BG / 1393 BG - U), positively polarized (HA / 45S5 BG / 1393 BG - P), negatively polarized (HA / 45S5 BG / 1393 BG - N)] and HA / 45S5 BG / 1393 BG – 30 NKN [unpolarized (HA / 45S5 BG / 1393 BG – 30 NKN U), positively polarized (HA / 45S5 BG / 1393 BG – 30 NKN -P), negatively polarized (HA / 45S5 BG / 1393 BG – 30 NKN -N)] composites in 24 well plates for 30 min. Subsequently, 500 µL growth media were added to each well and incubated further in CO_2 incubator. Media were changed every 48 – 72 h. All the experiments were performed in triplicates for 3 days, 5 days and 7 days.

3.15.1 Electrical stimulation

After 12 h of cell seeding, a parallel electric field of 1V/cm was applied on cultured samples for 5 min. using ScientiFic SMO702 digital oscilloscope. The similar process was repeated after 24 h of seeding [3.10]. The cells, seeded on glass disc and unpolarized surface was not treated with E-field and considered as control to compare results.

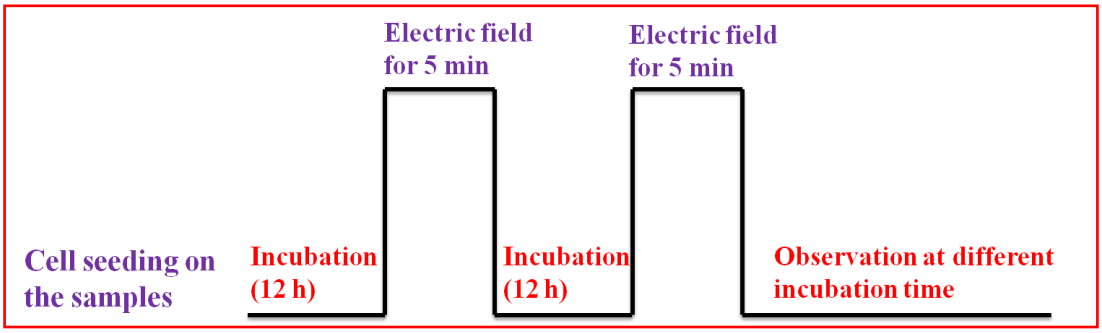

Fig. 3.10 Schematic representing the electrical stimulation sequence, adopted during osteogenic cell culture experiments.

3.15.2 Quantitative analyses (MTT assay)

Cell viability was assessed by MTT assay. The cells, seeded on HA / 45S5 BG / 1393 BG and HA / 45S5 BG / 1393 BG – 30 NKN composite were incubated for 3, 5, and 7 days in CO_2 incubator. After respective incubation period, the cells were washed with 1x PSB and 500 µL MTT (reconstituted 5mg/ml in 1x PBS) with DMEM without phenol red in 1:10 dilution was added and incubated further for 6 h to form formazan crystals. The crystals were dissolved in DMSO and optical density was measured at 590 nm using microplate reader (BioradiMark^{TM}).

3.15.3 Morphological analysis

Cells, seeded on HA / 1393 BG and HA / 1393 BG – 30 NKN samples were fixed in 4% paraformaldehyde at 4°C for 30 min, followed by permeabilization with 0.1% triton X 100 (in 1x PBS) for 10 min. Then blocking was performed using 1% Bovine Serum Albumin (BSA) in 1x PBS for 1 h at 4°C. After blocking cells were incubated with Alexa

Flour 488 Phalloidin (Invitrogen) diluted in 1% BSA in ratio of 1:500 for 1 h to stain cytoskeleton, whereas Hoechst dye (Invitrogen) were used to stain nuclei in dilution of 1:5000 with 1x PBS for 10 min. Proper washing (2-3 times) of the samples were done using 1x PBS after every steps. The stained cells were observed under fluorescent microscopy (Nikon Eclipse LV 100ND) for cell morphology and cell density quantification.

Summary

As a closure, this chapter demonstrated the synthesis of starting powder and fabrication of composite samples. To obtain higher densification, the compaction was done using CIP. Phase evolution and microstructural analysis were done using XRD, FTIR and SEM, respectively. Mechanical characterization (hardness, fracture toughness, flexural and compressive strength) and electrical measurement (dielectric constant, loss, ac conductivity and impedance analysis) were carried out to fabricate the composite comparable to natural bone. In addition, the antibacterial and cell culture tests in terms of quantitative as well as qualitative were performed to observe the effect of addition of piezoelectric NKN secondary phase in HA / 45S BG / 1393 BG matrix. Irrespective of addition of secondary phase, above measurements were performed to observe the relevancy of polarization surface charge and external electrical field on antibacterial and biocompatibility of fabricated composites.

References

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