Chapter 3

Materials and Methods

This chapter briefs the synthesis routes, adopted for the development of hydroxyapatite (HA), borate bioglass 1393 B3 (BBG), $BaTiO_3$ (BT), $CaTiO_3$ (CT), sodium potassium niobate (NKN) as well as HA- x ZnO (x = 3.0. 4.5 and 7.5 wt. %), HA- 30 vol. % NKN/BT/CT and BBG-30 vol. % NKN/BT composites. The phase evolution techniques such as X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and microstructural characterization (SEM and EDX) are performed to examine the phases and microstructure of developed compositions. In addition, the details of surface polarization conditions and protocols for antibacterial as well as cellular response are also described.

3.1. Synthesis

3.1.1. Synthesis of hydroxyapatite (HA)

Pure HA was synthesized using co-precipitation route [1]. In this process, commercially available calcium oxide (Merck = 90 %) and ortho-phosphoric acid (Loba Chemie, 85 %) were used as precursors. For the preparation of 25 gm batch, the dried CaO powder was weighed (15.48) gm and mixed with 750 ml of distilled water. The CaO solution was magnetically stirred at the temperature of 80 ºC. The aqueous solution of ortho-phosphoric acid was made by adding 9.5 ml of it to 1000 ml of distilled water. The obtained solutions were subjected to titration. This process takes nearly 4 h to complete. During titration, the temperature was maintained at 80 ºC and pH above 8 for the formation of phase pure HA. Ammonia Solution (NH_4OH) was added drop-wise to the solution during titration to maintain the pH value above 8. Usually, 5-10 ml of the above solution is required. After completion of titration, the obtained precipitate was aged overnight. The precipitate was filtered using filter paper to get sticky cake. The cake was

dried in air oven for 1 day at temperature of 100 ºC. After drying, cake was crushed using agate mortar and pestle to obtain HA powder [2]. Fig. 3.1 represents the synthesis flow chart for HA.

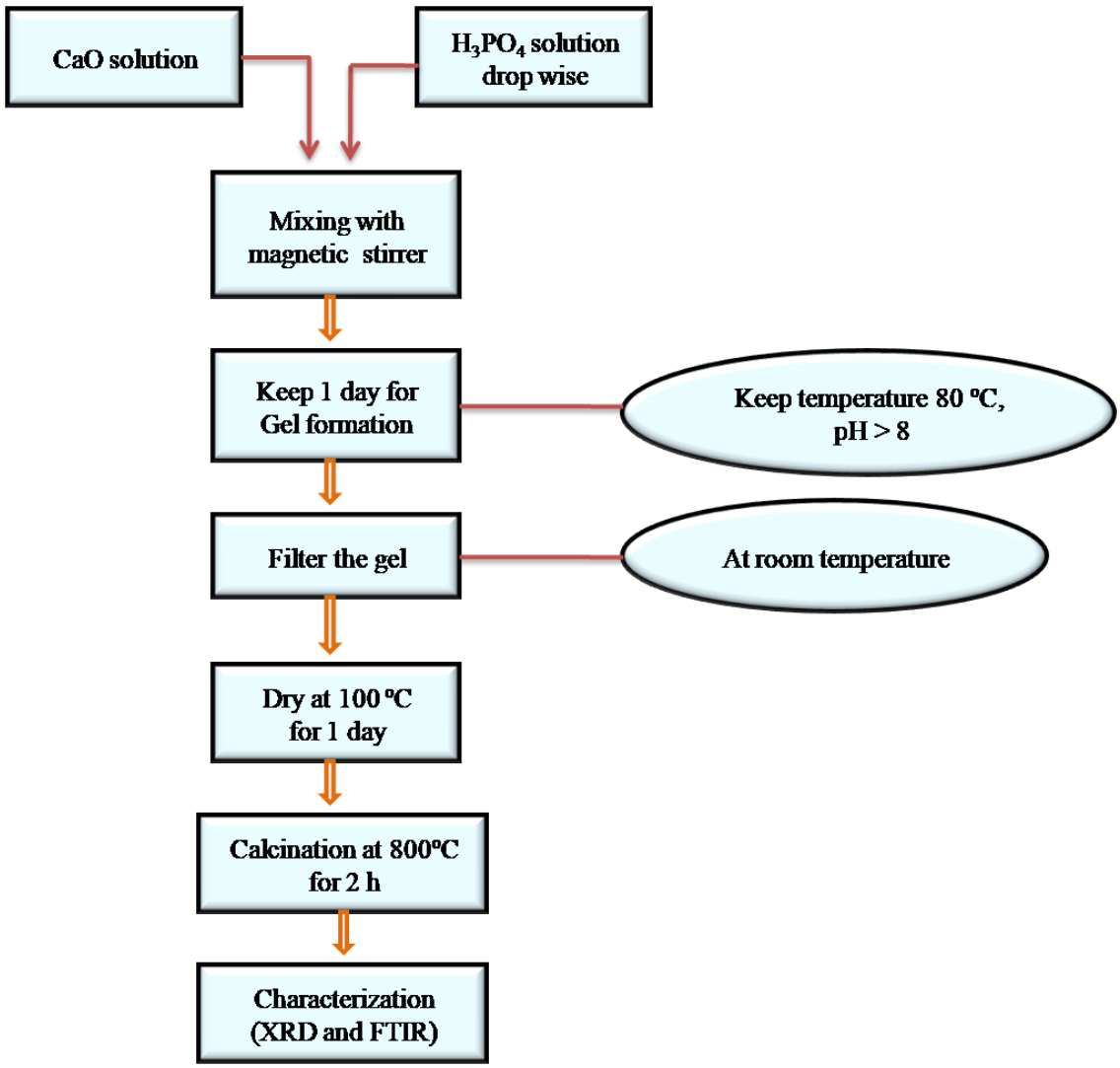

Fig. 3.1. Flow chart for the synthesis of Hydroxyapatite (HA)

The crushed powder was then calcined at 800 ºC for 2 h. Eq. 3.1 shows the formation of HA after calcination.

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

3.1.2. Synthesis of HA-xZnO composites

For the composite preparation, varying amounts of ZnO (3.0-7.5 wt. %) were blended with HA. Initially, the above-mentioned compositions were mixed using mortar and pestle for 30 min. It was then followed by ball milling using acetone and zirconia balls as supporting media (ball to powder ratio was kept at 1:4) for 16 h at 600 rpm. Further, the ball milled slurry was dried in air oven at 100 ℃ for 1 day and crushed again to get fine powders. Table 3.1 shows the composition and nomenclature for the prepared composites.

Table 3.1 Composition and nomenclature used for HA – ZnO composites

S.No.	HA (wt. %)	ZnO (wt. %)	Nomenclature
1.	97	3	HA-3 ZnO
2.	95.5	4.5	HA-4.5 ZnO
3.	92.5	7.5	HA-7.5 ZnO

3.1.3. Synthesis of barium titanate ($BaTiO_3$, BT)

BT was synthesized using solid state mixing route. In this process, $BaCO_3$ (Loba Chemie, 99 %) and TiO_2 (Loba Chemie, 99 %) were used as precursors. The stoichiometric amounts of $BaCO_3$ and TiO_2 powders were mixed in mortar and pestle for 30-60 min, followed by the ball milling for 24 h at 600 rpm. During milling, ethanol was used as wetting medium and zirconia balls were used as grinding media (ball to powder ratio, 1:4). After ball milling, the obtained semi liquid mixture was dried at100 ºC for 12 h, which was then powderised using mortar pestle. The obtained powder was calcined at 1000 ºC for 6 h.

3.1.4. Synthesis of calcium titanate ($CaTiO_3$, CT)

CT was synthesized using solid state mixing route. In this process, $CaCO_3$ (Loba Chemie, 99 %) and TiO_2 (Loba Chemie, 99 %) were used as precursors The

stoichiometric amounts of $CaCO_3$ and TiO_2 powders were mixed in mortar and pestle for 30-60 min, followed by the ball milling for 24 h at 600 rpm. During milling, ethanol was used as milling medium and zirconia balls were used as grinding media (ball to powder ratio, 1:4). After ball milling, the obtained semi liquid mixture was dried at 100 ºC for 12 h, which was then powderised using mortar pestle. The obtained powder was calcined at 1150 ºC for 6 h.

3.1.5. Synthesis of sodium potassium niobate ($Na_{0.5}K_{0.5}NbO_3$, NKN)

NKN was synthesized using solid state synthesis route. For this synthesis, Na_2CO_3 (Sigma Aldrich, 99%), K_2CO_3 (Sigma Aldrich, 99%) and Nb_2O_5 (Sigma Aldrich, 99.5%) were used as raw materials. The precursors were blended with ethanol media in polyethylene jar for 24 h using zirconia balls (ball to powder ratio, 1:4). Wet milled slurry was then poured into the beaker and stored for drying overnight in the oven at 100 ºC. To make the powder fine, the dried powder was crushed using the agate mortar pestle. The powder was then calcined at 910 ºC for 2 h. The formation of NKN after calcination occurs as,

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

3.1.6. Synthesis of HA- BT, HA- CT and HA- NKN composites

The composites of HA-x BT, HA-x CT and HA-x NKN (x = 30 vol. %) were synthesized using solid state synthesis route. The calcined BT, CT and NKN powders were mixed separately with HA powder and roll milled for 12 h at 600 rpm. During milling, ethanol was used as wetting medium and zirconia balls were used as grinding media (ball to powder ratio, 1:4). After ball milling, the obtained semi liquid mixture was dried at 100 ºC for 12 h, which was then powderised using mortar pestle. These composite systems were designated as HA- 30 BT, HA-30 CT, and HA-30 NKN, respectively.

3.1.7. Synthesis of borate bioglass (1393 B3)

Borate bioglass, 1393B3 (BBG) with composition of 56.6 % B_2O_3, 18.5 % CaO, 5.5 % Na_2O, 11.1 % K_2O, 4.6 % MgO, 3.7 % P_2O_5, was prepared using melt quench method. For this synthesis, MgO (Loba Chemie, 99 %), $CaCO_3$ (Loba Chemie, 99 %), Na_2CO_3 (Sigma Aldrich, 99 %), K_2CO_3 (Sigma Aldrich, 99 %), $(NH_4)\ H_2PO_4$ (Loba Chemie, 99 %) and boric acid (Loba Chemie, 99.5 %) were used as raw materials. Initially stoichiometric amounts of each regent were mixed in mortar pestle. Following this, the mixed powder was then melted in platinum crucible at 1300 ˚C for 2 h in an electric furnace. The melted glass was then quenched in deionized water at room temperature. The glassy samples were then ball milled to get the bioglass powder. The flow chart for the preparation of borate bioglass is shown in Fig. 3.2.

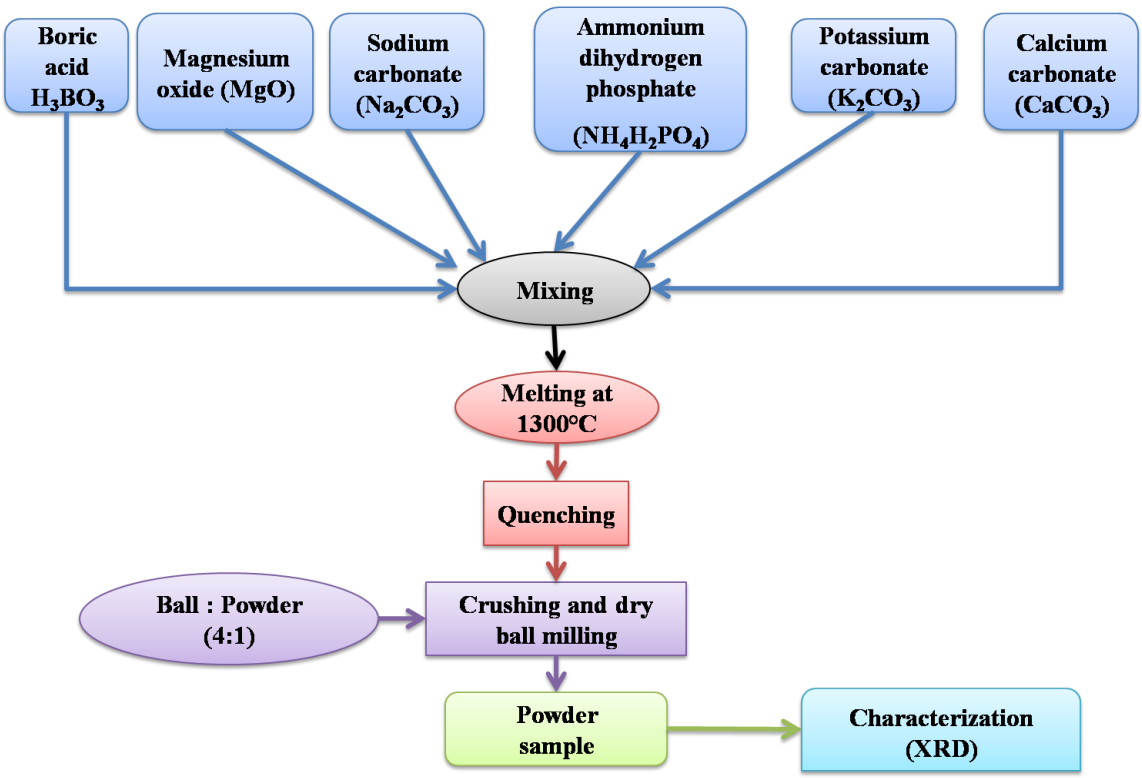

Fig. 3.2. Flow chart, representing the synthesis of borate bioglass 1393 B3 (BBG)

3.1.8. Synthesis of BBG- BT, BBG-NKN composite

The composites of BBG-x BT and BBG-x NKN (x = 30 vol. %) were prepared using solid state mixing method. The stoichiometric amounts of calcined BT and NKN powders were mixed separately with HA powder and roll milled for 12 h at 600 rpm. During milling, ethanol was used as milling medium and zirconia balls were used as grinding media (ball to powder ratio, 1:4). After ball milling, the obtained semi liquid mixture was dried at 100 ºC for 12 h, which was then powderised using mortar pestle.

3.1.9. Pelletization and sintering of processed samples

The pelletization of calcined powders was done using uniaxial hydraulic pressing at 5 MPa. The 12 mm diameter stainless steel die was used for preparation of pellets.

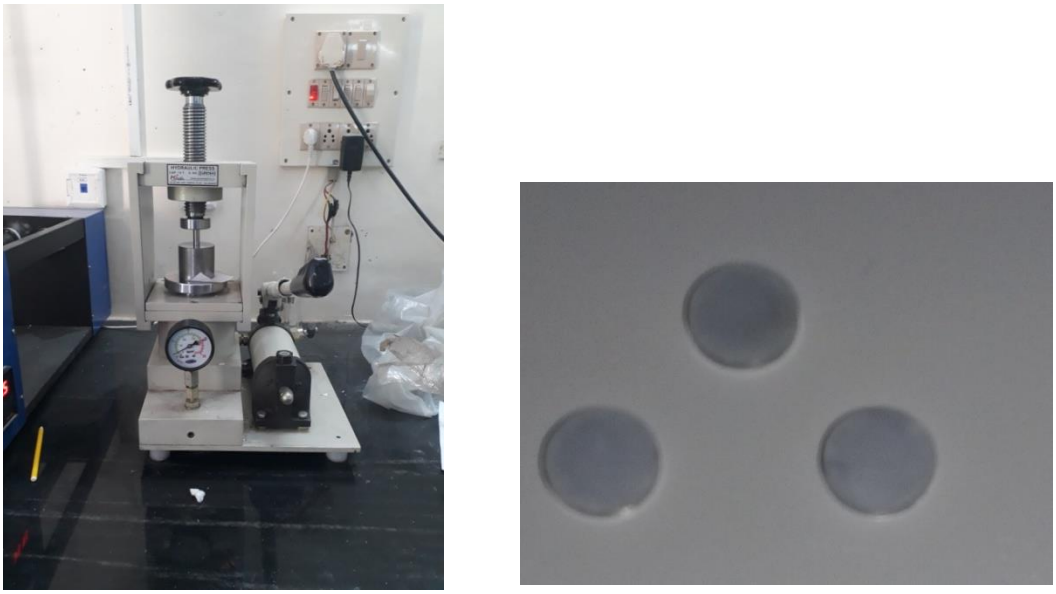

Fig. 3.3. Photographs of compacted samples prepared by uniaxial hydraulic press.

The prepared pellets were sintered at heating rate of 5 ºC/ min in high temperature (National Digital Furnace). Table 3.2 shows the sintering parameters for all the developed materials.

Table 3.2 Sintering parameters, optimized for the developed compositions

S.No.	Samples	Sintering temperature (°C)	Duration (h)
1.	HA	1200	2
2.	HA-x ZnO (x = 3.0, 4.5, and 7.5 wt. %)	1250	2
3.	BT and HA-30 vol. % BT	1300	2
4.	CT and HA-30 vol. % CT	1400	6
5.	NKN and HA-30 vol. % NKN	1075	2
6.	BBG and BBG-30 vol. % BT/NKN	650	0.5

3.2. Characterization

3.2.1. X-ray diffraction and Fourier transform infrared spectroscopic spectroscopy (FTIR)

The presence of phases in the sintered samples were examined using X-ray diffractometer (XRD, Rigaku Miniflex II Desktop X-ray Diffractometer) with Cu-Kα radiation (λ = 1.54056 Å). The diffraction angles were considered in the range of $20^{o} \leq 2\theta \leq 80^{o}$ with a step size of 0.05° and scan rate of 3° per minute. The XRD patterns of the sintered samples were then compared with the standard data in the JCPDS database using X pert high score software. The Fourier transform infrared spectroscopic (FTIR) was performed using FTIR Spectrometer (Bruker Model Tensor 27, Germany) in the wavelength range of 4000-400 cm^{-1}.

3.2.2. Scanning electron microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDX)

Scanning electron microscopy (SEM) was performed to observe the microstructure of fractured surfaces. For microstructural analyses, the samples were mirror polished and thermally etched at the temperatures 100 °C less than their respective sintering temperatures for 10 min and Au sputtered for 120 sec. SEM images were taken using

scanning electron microscope (SEM, Zeiss, EVO 18 Research). Energy dispersive X - ray spectroscopy (EDX) was used for elemental analyses.

3.3. Polarization treatment

The samples were polarized to examine the effect of surface charge on antibacterial as well as cellular response of prepared samples. The sintered and polished disc of (12 mm diameter and 1 mm thickness) all the developed compositions were poled using corona poling unit at polarizing temperature and voltage of 500 ºC and 20 kV, respectively (Fig 3.4). The samples were kept at 500 ºC for 30 min. then cooled to room temperature at constant exposure of electric field. The upper surface of the sample was negatively polarized (N-polarized) and the opposite side of the sample was positively polarized (P-polarized).

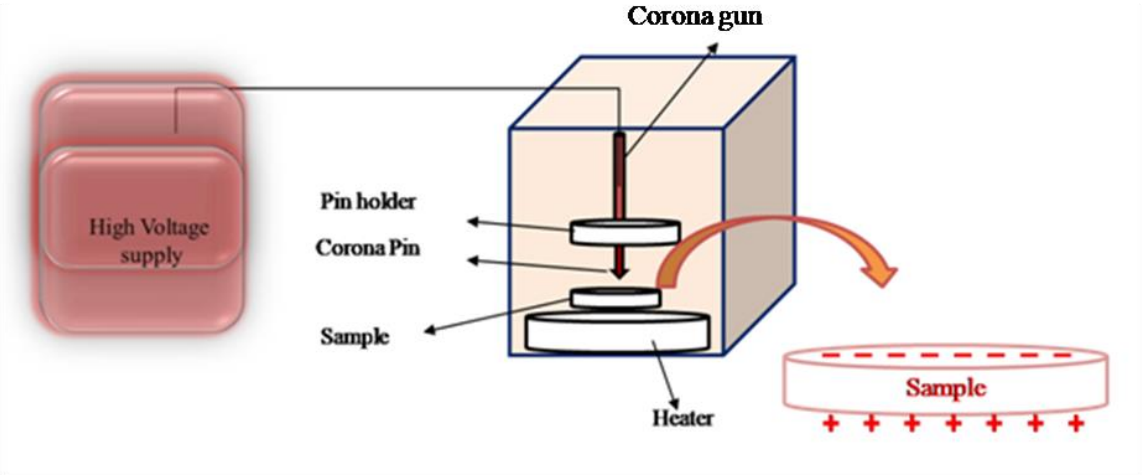

Fig. 3.4. *Schematic diagram represents the high voltage corona poling unit to develop the surface charge.*

3.4. Antibacterial response

For antibacterial assessment, *Escherichia Coli* (*E. coli*) and *Staphylococcus Aureus* (*S.aureus*) bacteria were used. Both the bacteria were obtained from Microbial Type Culture Collection (MTCC) Chandigarh, India. The received bacteria were revived in

nutrient broth (cultured media). The revived culture was diluted in media and absorbance of the diluted culture was taken using UV visible spectrophotometer, before seeding.

3.4.1. Quantitative analyses (MTT assay)

The antibacterial response of developed samples were analyzed quantitatively using MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The autoclaved [20 psi pressure and 121ºC temperature for 30 min] samples were washed twice with phosphate buffer saline (1x PBS) and dried. The dried pellets were seeded with 200 µL of bacterial culture in 24 well plates and incubated for 8 h at 37 ºC. After incubation, the culture media was removed and pellets were washed twice with 1x PBS. The equal amounts (500 µl) of reconstitute MTT (MTT: PBS in the ratio of 1: 10) was added in each well and further incubated at 37 ºC for 2 h. The MTT solution was replaced with solvent [(500µl of dimethyl sulfoxide (DMSO)] from each well to dissolve purple colour formazan crystals [3].The absorbance of formazan crystals is directly proportional to the number of viable cells [4]. The DMSO solution was then transferred in to a 96 well plate to measure the absorbance using ELISA microplate reader (Bio-red) at 595 nm. The statistical analyses of obtained results were done using SPSS 20 software. For statistical analyses of measured mean optical density, ANOVA method using Tukey test has been adopted at statistical significant value, $p < 0.05$.

3.4.2. Qualitative analyses (Live/dead assay)

Live/dead assay were performed to examine the effect of polarization on the population of bacteria on piezoelectric ceramics and electrets. Live and dead bacterial cells were stained using SYTO 9 and propidium iodide (PI) dyes, respectively. The bacteria cultured samples, after were washed twice with 1x PBS specific incubation period then mixture of both, SYTO 9 and PI dyes (1:1) were added (2-4 µl) for 30 min in dark. The samples were then observed under fluorescence microscopy (Nikon Eclipse LV 100

ND). Live/dead images were observed at the excitation/emission maxima of ~ 480/500 nm for SYTO 9 and ~490/635 nm PI dyes.

3.5. Cellular response

For in-vitro cellular response, human osteoblast like, SaOS2 and MG-63 cells were procured from national centre for cell science (NCCS) Pune, India. The SaOS2 cells were cultured in McCoy's 5A, supplemented with 1 % antibiotic and 15 % fetal bovine serum (FBS). Similarly, MG-63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with 15 % fetal bovine serum (FBS) and 1 % antibiotics. The cultured cells were incubated in humidified CO_2 (5 %) incubator (Thermo scientific Heracellvios 160i CO_2 incubator) at 37 ºC. After incubation, trypsin were added to detach the adhered cells from the flask wall, which was counted using hemocytometer. The sterilized samples were seeded with 10^4 cells and left for 30 min. in biosafety cabinet. Following this, 500 µL of respective growth media were added and incubated for 3, 5 and 7 days.

3.5.1. Quantitative analyses (MTT assay)

The quantitative analyses of *in-vitro* cellular response were performed using MTT assay. The viability of both, SaOS2 and MG-63 cells were calculated according to ISO 10993-5 standard. The dried samples were seeded with 10^4 cells/well (SaOS2 and MG-63) in 24 well plates with respective media and incubated for 3, 5 and 7 days in CO_2 incubator. After respective incubation periods, the samples were washed twice with 1x PBS and further incubated with reconstitute MTT (MTT: media in a ratio of 1:10) for 6 h to form the purple coloured formazan crystals. The formazan crystals were dissolved in DMSO and the optical density of viable cells was measured using ELISA micro plate reader. The viability of SaOS2 and MG-63 cells was calculated as [3],

$$\textbf{Cell viability} = \frac{\textbf{Mean absorbance of sample}}{\textbf{Mean absorbance of control}} \times \mathbf{100} \qquad \textbf{(3.3)}$$

3.5.2. Morphological analyses

The effect of polarization on morphology of adhered MG-63 cells was observed using fluorescent microscopy. The samples were taken in tissue culture plate and seeded with 10^4 cells/ well for 3 days in CO_2 incubator at 37 ºC. The culture media was replaced after 48 h. After desired incubation time, the cells were fixed (30 min) with paraformaldehyde (3.7 %). The permeabilization of cells was done using Triton X-100, followed by blocking with bovine serum albumin (BSA). The DAPI and Alexa Fluora 488 Phalloidin dyes were used to stain the nucleus and cytoskeleton of cells, respectively. The staining of cells was performed according to standard protocol. The stained cells were observed under fluorescent microscope (Nikon Eclipse LV 100 ND).

3.6. Closure

This chapter demonstrated the processing routes, adopted for the development of HA, BT, CT, NKN, borate bioglass 1393 B3 and HA-x ZnO (x = 3.0, 4.5, and 7.5 wt. %), HA- 30 vol. % BT/CT/NKN, BBG-30 vol. % BT/NKN composites. The processing of HA and BBG was done by co-precipitation method and melt quenching route, respectively. However, BT, CT, NKN and their composites were prepared using solid state sintering route. The pelletization of calcined material was done by uniaxial hydraulic press. The optimized sintering parameters were also mentioned. Phase and microstructural characterization techniques such as XRD, FTIR and SEM, were briefly discussed. This chapter also discussed the polarizing parameters to develop the charge on sample surface. In addition, the standard protocol for antibacterial as well as cellular response has been elaborated.

References

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