CHAPTER 3

MATERIALS AND METHODS

1. MATERIALS AND METHODS

3.1. Materials

3.1.1. Nanoparticle synthesis

Copper sulfate pentahydrate ($CuSO_4.5H_2O$) (102790) and Sodium chloride (NaCl) (59625) were purchased from Merck, India. Starch (101257) and Calcium hydroxide ($Ca(OH)_2$) (C7887) were procured from Sigma Aldrich Chemical, Mumbai, India. Orthophosphoric acid (H_3PO_4) (1.00565), chloroform ($CHCl_3$) (528730), ammonia solution (NH4OH) (1.05422), and hydrochloric acid (HCl) (2104) were obtained from Merck Ltd. Mumbai, India. Bovine Serum Albumin and Bradford's reagent were purchased from Merck, India. L-ascorbic acid was purchased from Sigma- Aldrich, US. Triethyl phosphate (TEP) (GRM3766) was purchased from HiMedia. Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) (1.93708.0521) and Nitric acid (HNO_3) (1.93006.0521) were all purchased from Merck. Tetraethyl Orthosilicate (TEOS) (131903) was purchased from Sigma Aldrich. Absolute ethanol (C_2H_5OH) 99.9% was used for nanoparticle synthesis and it was purchased from Merck. Graphene oxide nanoparticles (15-20 sheets) (796034) were procured from Sigma Aldrich. Distilled water and Milli-Q water were used for nanoparticle synthesis.

3.1.2. Preparation of Scaffold

Chitosan medium molecular weight (Ch) (448877) and Gelatin (G) (G9382) were all obtained from Sigma. β-Tricalcium Phosphate (β-TCP) (49963) was procured from Sigma Aldrich Chemical, Mumbai, India. Lysozyme (L6876) was purchased from Merck Ltd. Mumbai, India. N-(3- Dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (EDC) (RM1817), N-Hydroxysuccinimide (NHS) (RM1120), Glutaraldehyde (RM5927) and acetic acid glacial (AS119) were purchased from HiMedia. Distilled water and Milli-Q water were used for scaffold preparation.

3.1.3. Osteoblast cell culture studies

For the cell culture studies, MG-63 cell lines were procured from NCCS Pune, India. Trisodium citrate dihydrate (S1804), and phosphate buffer saline (DPBS) (D8537) were purchased from Merck Ltd. Mumbai, India. Sodium pyruvate (Pyruvic acid sodium salt) (PCT0503) was purchased from HiMedia. Dulbecco Minimum essential medium (DMEM) (12-604F) with high glucose was procured from Lonza. Live/Dead cytotoxicity kit for mammalian cells (L3224) was purchased from HiMedia. Alkaline phosphatase (ALP) (RM4006) was purchased

from HiMedia. ß-glycerophosphate and Tris buffer were also procured from HiMedia. p-nitrophenyl phosphate (pNPP) (p-nitrophenyl phosphate Tablets) (N9389) was procured from SIGMAFASTTM. Cetylpyridinium chloride and Alizarin Red Stain were purchased from HiMedia, India. 4′,6-diamidino-2-phenylindole (DAPI) dye (D9542) was procured from Sigma. 4′,6-diamidino-2-phenylindole (DAPI) dye (D9542) was procured from Sigma. Acridine Orange (AO) (TC262), Propidium Iodide solution (PI) (ML067) and Triton-X 100 (TC286) were purchased HiMedia. MTT 3-[4,5-dimethyl-thiazol-2-yl]- 2,5-diphenyltetrazolium bromide) assay kit (TC151), paraformaldehyde (GRM3660), and Dimethyl sulfoxide (DMSO) (TC151) were also procured from HiMedia, India. Collagenase-I, Glutamine solution and DCFDA- Cellular Reactive Oxygen Species Detection Assay Kit were also purchased from HiMedia (Mumbai, India). Sodium bicarbonate (Na2(CO3)2) was purchased from Merck. Trypsin-EDTA solution (TCL042) was purchased from HiMedia. Fetal bovine serum (FBS) (RM9955) and Pen-strep solution (antibiotic solution) (PS) (A001) were purchased from HiMedia, India. Cell Viability Assay Kit *Green/Red Dual Fluorescence* was purchased from AAT Bioquest®. ECIS® (Electric Cell-substrate Impedance Sensing) and 81E array from Applied BioPhysics were used for this study. Absolute ethanol (C2H5OH) 99.9% was used for all the cell culture experiments and it was purchased from Merck. Throughout the experiments, Distilled water and Milli-Q water were utilized.

3.2. Methods

3.2.1. Synthesis of Copper Nanoparticles (CN) for the development of nanocomposite scaffold

As discussed earlier, the addition of metal ions shows improved wound healing properties and mechanical properties of the scaffold. Therefore, the synthesis of copper nanoparticles is carried out in this section. Since, chemical reduction method is easy, cost-effective and efficient, and it can apprehend improved size and size dispersion control by optimizing the experimental factors therefore it was adopted. CN was synthesized by using a chemical reduction approach in which copper (II) sulfate pentahydrate was used as a precursor salt while starch was also added as a capping agent (Khan A et. al.,2016; Cushing, B.L. et al.,2004). The capping agents or stabilizers can significantly reduce the oxidation but may not prevent it completely because of their molecular motion. Firstly, 100mL of 0.1M copper (II) sulfate pentahydrate solution was added into 1.2% of 240 mL of starch solution along with vigorous stirring for 30 min followed by the second step in which 100ml of 0.2Mascorbic acid solution was added to the previous solution under constant stirring. Subsequently, 60mL of 1M

sodium hydroxide solution was added to the above prepared solution followed by undisturbed stirring and heating at 80°C for 1.30 h. A colour change was observed from yellow to ocher colour after it (Khan et al., 2016). Fig.3.1 shows the process of copper nanoparticle synthesis. Thereafter the reaction was completed and the solution was taken up from the heat and it was allowed to settle overnight. After 24h, the supernatant was vigilantly discarded and the precipitate was separated from that solution by centrifugation at 10000rpm. Then the precipitate was washed using 70% ethanol and deionized water three to four times to take out the excessive starch bound with the nanoparticles. Ocher colour precipitate obtained was dried in a vacuum oven at rt. After drying the nanoparticles, they were stored for further experiments.

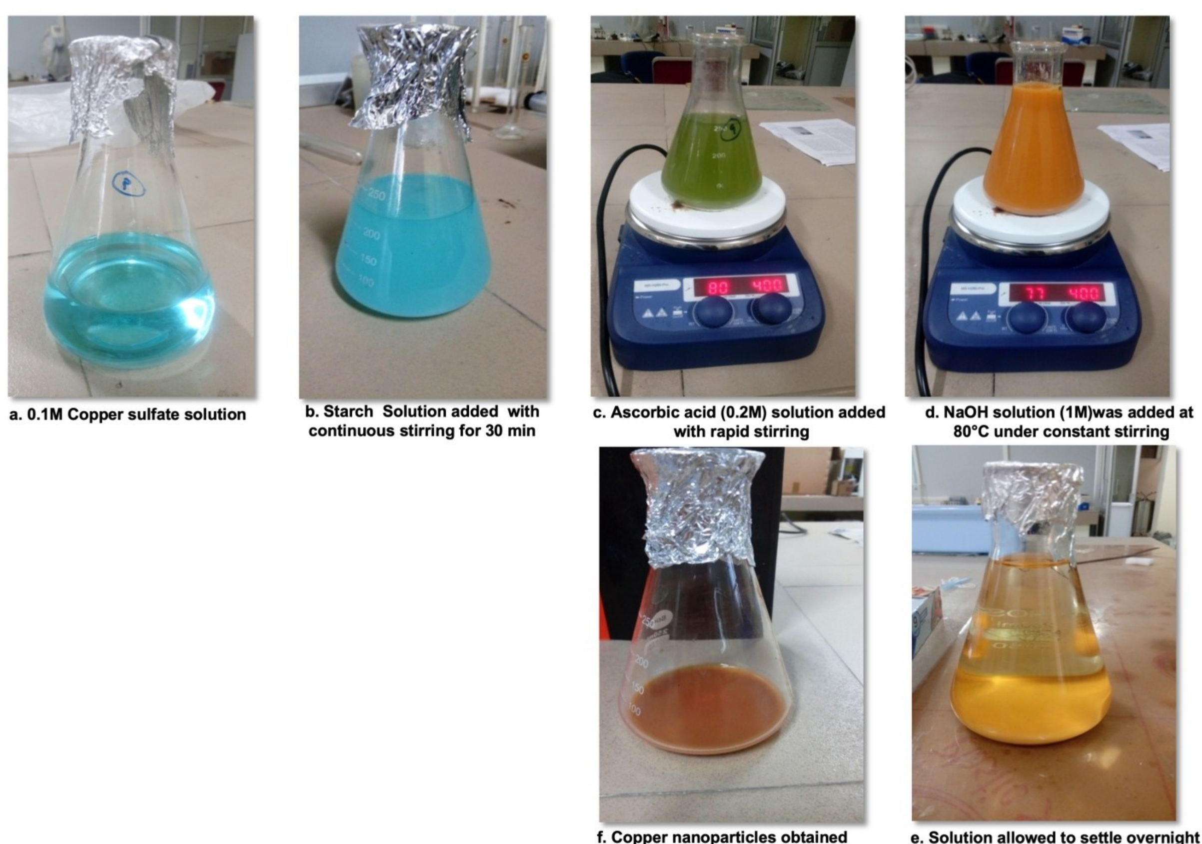

Figure 3.1: Synthesis of Copper Nanoparticles through chemical reduction approach.

3.2.2. Preparation of Chitosan/Gelatin Scaffold using Lyophilization

2% w/v chitosan solution was prepared in 98mL double distilled water (DDW) followed by the addition of 2% glacial acetic acid(v/v). 7% w/v gelatin solution was prepared by dissolving 7 gm of gelatin in 100 mL of distilled water at constant stirring (Kumar et al.,2017). These solutions were mixed on a hot magnetic plate at 400rpm and further added with corresponding nanoparticles for the development of desired polymeric solutions. These solutions were casted

in DPS tubes and they were kept at -80°C overnight. After 24 h, the frozen polymer blend solutions were lyophilized at -50°C temperature using a Labconco lyophilizer. Lyophilization process was used to achieve porous scaffold as discussed previously in scaffold fabrication techniques. These scaffolds were further crosslinked with EDC-NHS (3 w%) in a 2:1 (w/w) ratio prepared in ethanol: water [95:5 (v/v)] solution to improves their stability. Then washed with DW thrice and were lyophilized again for further study.

3.2.3. Fabrication of CN-embedded Chitosan/Gelatin scaffolds

Chitosan was dissolved in 1% of 1M CH_3COOH (acetic-acid) aqueous solution to form a 2% polymer solution with constant rapid stirring at 37°C temperature. Thereafter 7% polymer solution of gelatin was made by dissolving 7gm of gelatin in 100mL of distilled water at constant stirring. 3samples were made with different compositions of chitosan and gelatin. These samples were poured into DPS tubes and frozen at -80^{0}C overnight. After 24 h the frozen polymer blend solutions were lyophilized at -50°C temperature using a Labconco lyophilizer. Table No.3.1 shows the composition used for preparing the scaffolds without CN and with CN. The copper-chitosan/gelatin scaffolds were prepared by adding slowly a blend of 2 % w/v chitosan solution and 7% w/v solution of gelatin to 50 mL 0.01% copper, then 0.02% and finally 0.03% copper solution (Kumari et. al.,2019). Fig.3.2 shows the development of copper nanoparticles incorporated scaffolds. The mixture was vortexed for 5 minutes for homogeneity and was poured into DPS tubes and further incubated overnight at - 80°C and later freeze-dried to completely remove the solvent. The addition of chitosan/gelatin solution to the copper solution led to the colour change of the chitosan and gelatin solution to a greenish colour (Kumari et al.,2019).

Table No. 3.1 Composition of developed scaffolds

Scaffolds	**Chitosan (Ch) (%)**	**Gelatin (G) (%)**	**Copper Nanoparticles (CN) (%)**
Ch-G1 (F1)	50	50	N/A
Ch-G2 (F2)	25	75	N/A
Ch-G3 (F3)	75	25	N/A
C-G-Cu1 (F4)	50	50	0.01
C-G-Cu2 (F5)	25	75	0.02
C-G-Cu3 (F6)	75	25	0.03

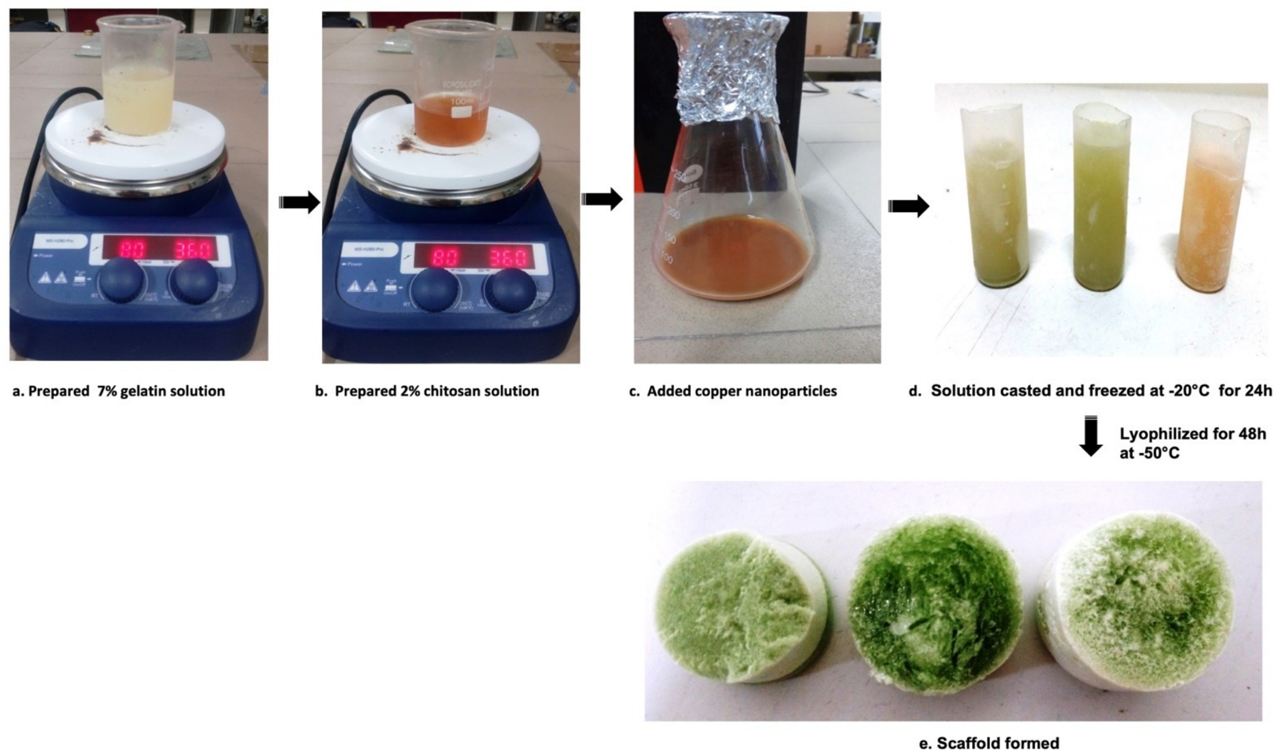

Figure 3.2: Fabrication of CN based scaffolds for tissue engineering. Figure 3.2(a): Preparation of 7% gelatin solution. Figure 3.2(b): Preparation of 2% chitosan solution. Figure 3.2(c): Addition of copper nanoparticles in different concentrations to the chitosan- gelatin blend. Figure 3.2(d): 0.0%1, 0.03% and 0.02% CN incorporated Chitosan-gelatin solution respectively from left to right. Figure 3.2(e): 0.0%1, 0.03% and 0.02% CN incorporated Chitosan-gelatin scaffold respectively from left to right.

3.2.4. Synthesis of Nano-hydroxyapatite using Sol-gel method

For the preparation of Ch/G scaffolds incorporated with CN/nHAP for improved mechanical strength and controlled degradation, we synthesized nanohydroxyapatite using the sol-gel method reported in a previous study (Kalaiselvi et al.,2017). For the synthesis of nHAP, orthophosphoric acid (H_3PO_4) and calcium hydroxide Ca $(OH)_2$ were used as a precursor. 0.6M H_3PO_4 was prepared in double distilled water (DDW) and 1M of Ca $(OH)_2$ was also prepared in DDW by stirring it continuously for 30min at 400rpm at rt. Thereafter the phosphoric acid solution was added dropwise into the calcium hydroxide solution. This solution was kept under constant stirring for 1h and the pH was maintained at 11 by the addition of ammonia solution to it. The procured sol was aged for 24h and the precipitate derived was dried at 180°C for 6h in a hot air oven. The final product was obtained after sintering the dried precipitate at 600°C for 3h in a muffle furnace (Kalaiselvi et al.,2017). This powder was stored for further application in a vial.

3.2.5. Development of CN/nHAP infused Ch/G based nanocomposite scaffolds

2% chitosan solution, 8% gelatin solution, and 10mL copper nanoparticles solution were prepared separately in 2% acetic acid, and double distilled water respectively. Then 3.5% nHAP was mixed to a 1:1 ratio of 10mL Ch-G and the mixture was continuously stirred at 400rpm to form a uniform polymeric solution. Above 3.5% nHAP, the composite was not blending and precipitating therefore, a 3.5% concentration of nHAP was chosen for the experiment. After that 0.01%, CN was added to the above-mentioned solution and this solution was ultrasonicated for 20-30min for appropriate mixing of the solution. This solution was cast in cylindrical tubes and stored at -80°C for 48 h. Finally, the porous composite scaffolds were fabricated by lyophilization of the nanocomposite solution at 40mm torr and -40°C (Kumari et al.,2023). The same process was repeated for 0.02% and 0.03% CN-based scaffolds. Ch-G-nHAP scaffolds served as a positive control for the Ch- G-nHAP-CN samples.

For CN-based scaffolds, firstly the nanoparticles were dispersed and sonicated in DDW for 23h. The composition of the prepared scaffolds is summarized in Table no. 3.2. For crosslinking the fabricated scaffolds, the dried scaffolds were firstly crosslinked with EDC-NHS (3 wt%) in 2:1 (w/w) ratio prepared in ethanol: water [95:5 (v/v)] solution (Bhisham et al.,2019). Then washed with DW thrice and were lyophilized again for further study. The developed composites were tested *in-vitro* for morphological, physicochemical and mechanical characterization.

Table No. 3.2 Composition of nHAP and CN incorporated scaffolds

S.No.	Sample	Chitosan (C) (%)	Gelatin (G) (%)	Copper Nanoparticles (CN) (%)	Nano hydroxyapatite (nHAP) (%)	Total Volume (mL)
1.	**C**	**2**	**8**	**0**	**0**	**10**
2.	**C-G**	**2**	**8**	**0**	**0**	**10**
3.	**C-G- nHAP**	**2**	**8**	**0**	**3.5**	**10**
4.	**C-G-nHAP-0.01CN**	**2**	**8**	**0.01**	**3.5**	**10**
5.	**C-G-nHAP-0.02CN**	**2**	**8**	**0.02**	**3.5**	**10**

6.	C-G-nHAP-0.03CN	2	8	0.03	3.5	10

3.2.6. ECIS study for osteoblast cell culture

ECIS is based on the principle of application of a small non-invasive alternating current (I) which is applied across the electrode pattern at the bottom of 81E arrays. It results in a developed potential (V) across the electrodes which is measured by the ECIS instrument. The impedance (Z) is measured by Ohm's law as illustrated in equation 1. For this study, the MG-63 cells were procured from ATCC, USA. The cells were cultured in DMEM medium and 10^5cells/cm^2 were taken further for the seeding of cells in the scaffolds. The cell suspension was added to the 81E arrays where we placed the sterilized scaffolds cut in 2mm sections carefully which were attached to the electrodes. Thereafter, we placed the electrode array with the scaffold and seeded cells into a humidified incubator at 37°C with 5% CO_2 to allow the cells to attach to the scaffold and start growing. Initial attachment may take several hours to overnight. After this the electrode array was connected to the ECIS device and it was ensured that all connections were secure and that the setup was stable. Then, we continuously monitored the impedance for 45 hours. The cells acted as insulators which led to an increase in the impedance. With the growth of cells, the electrodes got covered, and the current was impeded in a way that was related to the number of cells that covered the electrode, their morphology and the nature of cell attachment. After the cells were stimulated to alter their function, the impedance was also altered due to the accompanying modifications in the MG-63 morphology. Fig.3.3(a-b) shows the ECIS system and the mechanism involved in analyzing the cell behaviour of osteoblast cells. The data obtained was impedance vs time. The experiment was conducted at a low frequency of 4000Hz to view the cell-cell interaction and cell-substrate interaction. This study was performed at the Department of Biomedical Engineering, FIU, Miami as per standard protocol.

$Z = V/I$--Eq.1

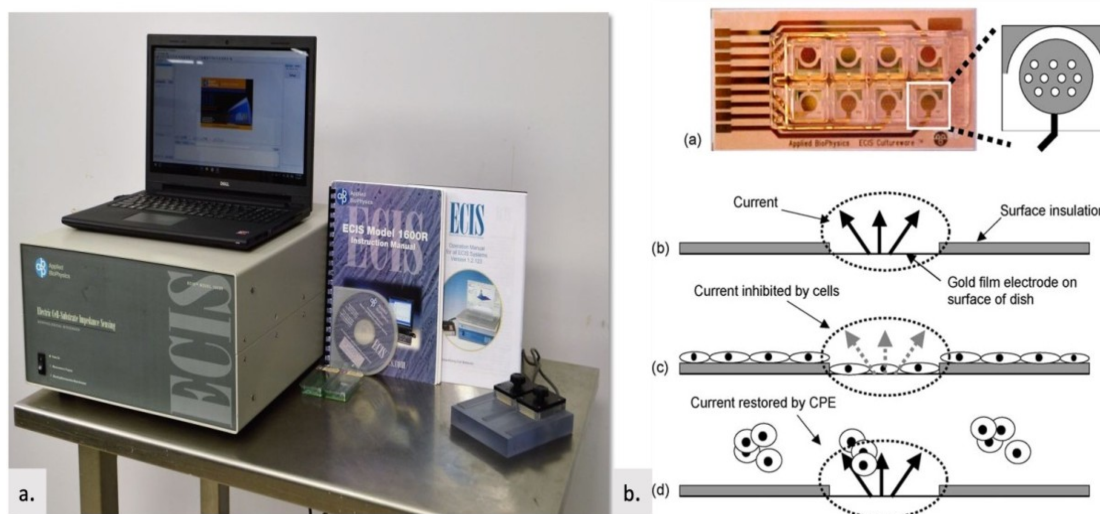

Figure 3.3(a)- ECIS system for monitoring cell behaviour.
Fig.3.3(b)-Mechanism involved for ECIS study of cell behaviour (Source- Applied Biophysics)

3.2.7. Synthesis of Nanobioglass using sol-gel method for fabrication of NBG-based Ch/G scaffold

Nanobioglass was synthesized by sol-gel method (acid-mediated) with the molar composition of 60%SiO_2, 4% P_2O_5 and 36% CaO (Vichery et al.,2016). For this process, 4.53mL TEOS was dissolved in distilled water followed by the addition of 3.022gm Ca $(NO_3)_2.4H_2O$ into it. Then 0.195mL TEP was slowly added to it. For preparing a transparent sol, the acidic pH was adjusted by adding 2M HNO_3. Then finally, the solution was kept under stirring for a further 5-6 h for gel formation. After that, the sol-gel was kept at 60°C inside the hot air oven for the gelation process to occur followed by the ageing for the next 3-4 days. Thereafter the synthesized nanobioglass was sintered at 600°C for a further 2 h. The obtained nanobioglass powder was stored for further application.

3.2.8. Development of NBG and β-TCP infused Ch/G based nanocomposite scaffold

8% Gelatin solution was prepared (100 mL distilled water) in which 2% chitosan solution (made in 100 mL DW) was mixed. Followed by the addition of 6% nanobioglass to 20 mL of the total Ch-G solution which was kept under constant stirring at 400 rpm. Similarly, 6% ß-TCP was mixed in the 20 mL Ch-G polymer solution with constant stirring. Another composition with a 1:1 ratio of ß-TCP and NBG was also prepared by adding 6% ß-TCP and 6% NBG to 20mL Ch-G solution with continuous mixing at room temperature (Kumari et al.,2023). Table 3.3 shows the composition of the developed scaffolds. These samples were stored at - 21°C in the refrigerator for 24h and then they were lyophilized at -40°C under vacuum for 48h. After 48h the developed scaffolds were crosslinked in the EDC-

NHS/Glutaraldehyde solution as discussed previously for Ch/G- Cn/nHAP scaffolds (Bhisham et al.,2019). The scaffolds were incubated in the solution for a further 2 h. Followed by washing with 70% ethanol solution, 50% ethanol solution, 10% ethanol solution and distilled water simultaneously to remove any excess crosslinking solution. Fig.3.4 shows the formation of nanocomposite scaffolds with NBG and ß-TCP. A control Ch-G scaffold was also prepared without ß-TCP or NBG powder.

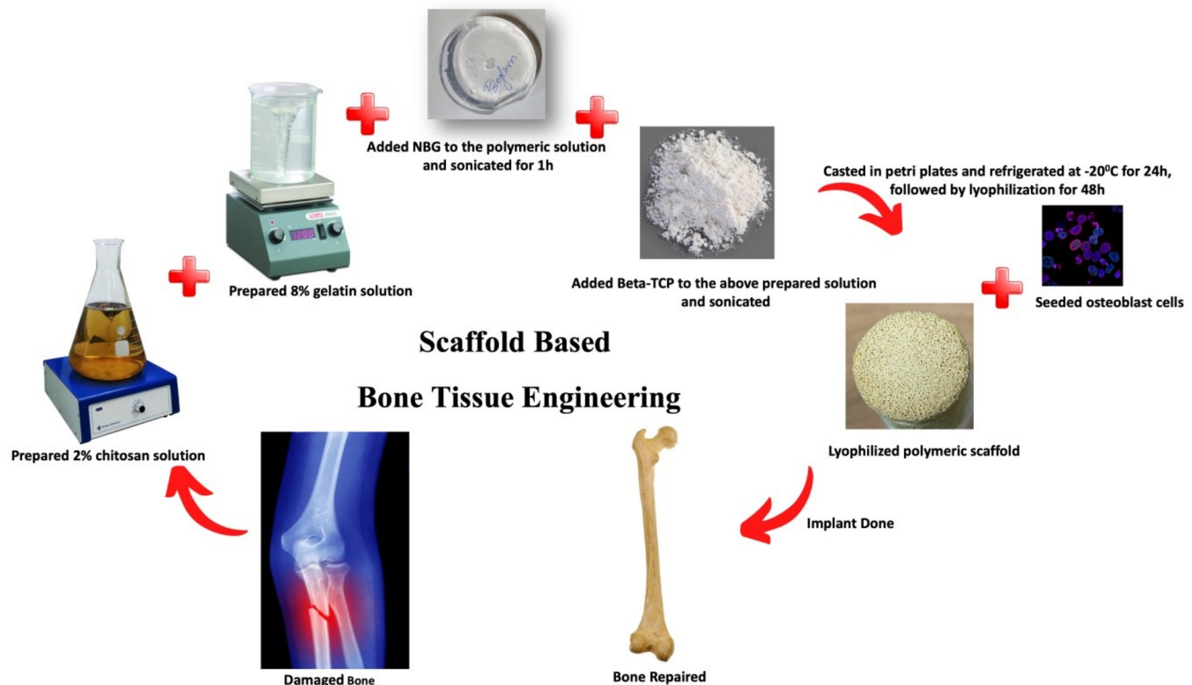

Figure3.4: Fabrication of NBG and TCP reinforced Chitosan-gelatin scaffolds for Bone tissue engineering application

Table No. 3.3 Composition of NBG and β-TCP infused Ch/G scaffolds

S.No.	Sample	Chitosan (%)	Gelatin (%)	Nanobioglass :Tricalcium Phosphate	Total Volume (mL)
1.	**Ch-G**	**2**	**8**	**0:0**	**10**
2.	**Ch-G-NBG**	**2**	**8**	**1:0**	**10**
3.	**Ch-G-TCP**	**2**	**8**	**0:1**	**10**
4.	**Ch-G-NBG/TCP**	**2**	**8**	**1:1**	**10**

3.2.9. Preparation of Crosslinking solution for NBG and β-TCP-infused Ch/G scaffold

For preparing the EDC-NHS/Glutaraldehyde crosslinking solution, firstly, ethanol solution was prepared in 95:5 (V/V) ratio. Followed by adding 3% (Wt) of EDC: NHS in 2:1 ratio (Wt/Wt) to the above solution (Bhisham et al.,2019). Chemical crosslinking has been

performed using carbodiimide chemistry which involves both EDC/NHS and glutaraldehyde solution for the crosslinking of the polymer chain to ensure better stability of the polymeric scaffold (Funakoshi et al.,2015). The unused solution was kept in the refrigerator for further application. 0.5% glutaraldehyde was further added to the solution after soaking the scaffolds for 24h in the EDC-NHS solution to complete this process. The scaffolds were taken out from this solution and were vacuum-dried for further studies.

3.2.10. Fabrication of Graphene oxide (GO) and NBG embedded Ch/G nanocomposite scaffold

The freeze-drying technique was used to develop Ch/G nanocomposite scaffolds embedded with graphene oxide (GO) and NBG nanoparticles. Scaffolds were prepared in different compositions, as mentioned in Table 3.4. Chitosan (C) and gelatin (G) solutions were prepared. The percentage of chitosan solution was fixed to 2%(w/v), and gelatin solution was selected to 8%(w/v). Firstly, 2gm of Chitosan was dissolved in 100mL DW followed by addition of 0.2% GAA to prepare 2% chitosan solution. 8% gelatin solution was also prepared by dissolving 8gm gelatin in 100mL distilled water. The percentage of NBG was set to 8%(w/v) for the experiments as reported from previous studies, and percentage of GO nanoparticles was varied in three different ratios (Bhisham et al.,2021). Gelatin solution was added to the chitosan solution, and it was kept at room temperature on a magnetic stirrer with constant stirring at 200 rpm. Once the blended mixture appeared uniform, NBG nanoparticles were added slowly with continuous stirring for 4h. Different concentrations of Graphene oxide (30mg, 60mg and 90mg) were firstly dispersed in 100mL water followed by ultrasonication for 2-3h duration. After the GO solution was prepared in water, it was added to the composite solution at constant stirring. After 2h, this solution was cautiously cast and was refrigerated at -80°C overnight, followed by lyophilization at -40°C for 48 h (Kumari et al.,2022). Fig.3.5 shows the development of Ch/G scaffolds incorporated with GO and NBG nanoparticles. The dried scaffolds were firstly crosslinked with EDC-NHS (3wt%) in a 2:1 (w/w) ratio prepared in ethanol: water [95:5 (v/v)] solution for enhancing the stability of the scaffolds. Then washed with 70% ethanol solution, followed by washing with 50% ethanol solution and then 20% and 10% ethanol solution. Finally, they were washed with DW to remove any excess crosslinking solution over the surface and were lyophilized again. The C-G-NBG scaffolds without GO nanoparticles were taken as a positive control for the study.

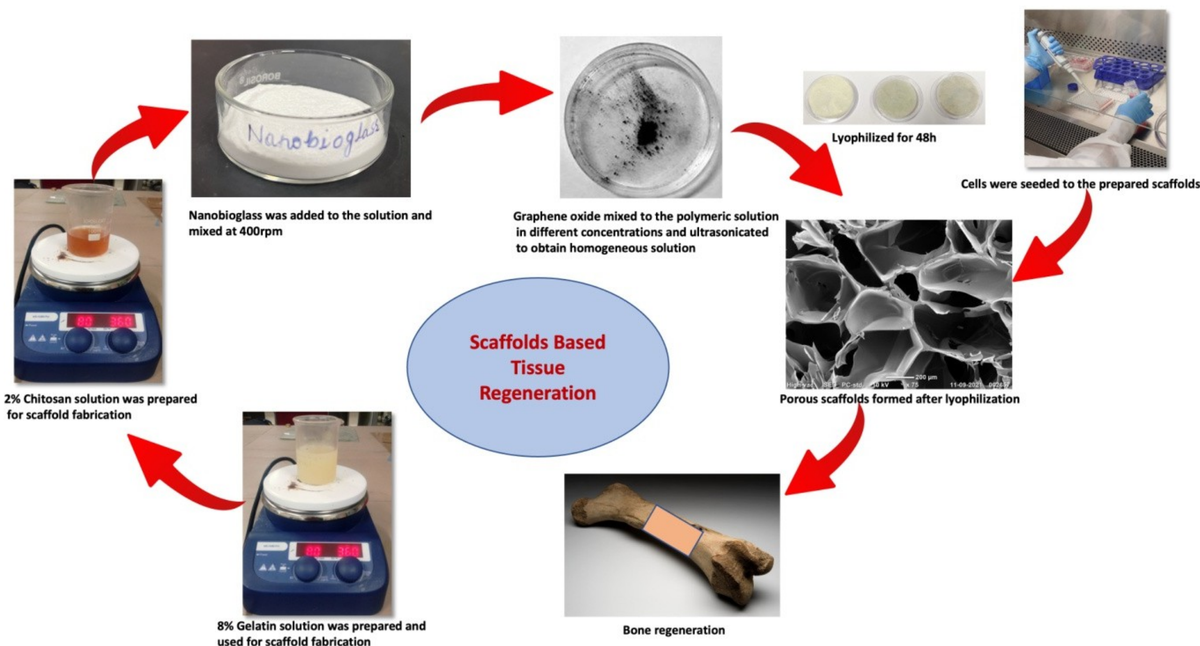

Figure 3.5: Fabrication of GO and NBG incorporated Chitosan and gelatin based scaffolds

Table No.3.4 Composition of NBG and GO incorporated scaffolds

Samples	Chitosan (Ch) (%) (w/v)	Gelatin (G) (%) (w/v)	Nano-bioglass (NBG) (%) (w/v)	Graphene Oxide nanoparticles (GO) (%) (v/v)
Ch-G	2	8	0	0
Ch-G-NBG	2	8	12	0
Ch-G-NBG-30%GO	2	8	12	30
Ch-G-NBG-60%GO	2	8	12	60
Ch-G-NBG-90%GO	2	8	12	90

3.3. Characterization of Nanoparticles (copper, nanohydroxyapatite, nanobioglass, graphene oxide)

3.3.1. Morphological and elemental analysis of CN, nHAP, NBG, GO nanoparticles

Surface morphology of the synthesized nanoparticles was examined by scanning electron microscopy (SEM, ZEISS-EVO18) combined with an energy-dispersive X-ray spectrometer (EDX) (6490 LA) to understand the formation of these nanoparticles. Sample preparation for SEM was done in powdered form and since the nanoparticles were already dried therefore same

powder was used for analysis (Mohan et al.,2016). Only a small pinch of nanoparticle powder was dispersed onto a double-sided adhesive carbon conductive tape and it was coated using golden sputter coating via an ion sputtering device (JFC 1500). This was placed on the sample holder to allow the beam to fall on its surface. EDX was carried out at an acceleration voltage of 20.0 kV. The same sample of SEM was used to study the elemental composition both in normalized atomic% concentration as well as in normalized weight concentration (Mohan et al.,2016).

High-resolution scanning Electron Microscopy (HRSEM FEI NovananoSEM 450) was also performed to observe the size and morphology of the synthesized nanoparticles at a higher resolution. Nanoparticle powder was firstly dispersed in 5mL ethanol and 5mL water in a 1:1 ratio after that this solution was sonicated for 1-2 hours to avoid agglomeration of the nanoparticles. After proper separation of particles 2µl of the suspension was taken on a platinum film and it was allowed to dry at room temperature. Once the suspension was dry, HRSEM characterization was done.

3.3.2. Structural and Functional Characterization of CN, nHAP, NBG, GO nanoparticles

XRD analysis of synthesized nanoparticles was performed using a Rigaku-MiniFlex 600 diffractometer that was operated at 40kV and 30mA with Cu Ka radiation (1.54Å) as a source. The sampling time was 0.5s and the measurement temperature was 25°C. The scanning range of 2θ was between 20° C and 90°C (Sagadevan et al.,2015). Sample preparation for XRD was easy as the nanoparticles were synthesized in a powdered form. The nanoparticle powder was placed on top of an aluminium slide and it was ensured that the powdered nanoparticles were spread out properly to cover up the specified area. The mean size of nanoparticles was measured from the broadening of the diffraction peaks. According to the JCPDS database, these peaks indicated intensive reflections.

Transmission electron microscopy was done to characterize the size, shape and morphology of synthesized nanoparticles using the FEI-TECNAI G220 TWIN instrument. Sample preparation for TEM was a quite sensitive process. Since the nanoparticles were in powdered form therefore firstly a small tinge of powdered nanoparticles was immersed in ethanol which was used as a solvent. Further, this solution was sonicated for 30-40 min using an ultrasonicator to ensure proper dispersion of the nanoparticles. Once the suspension was prepared, 2µl-5µl of this suspension was poured onto the grid towards the mesh side and it was properly smeared and allowed to dry at room temperature. The sample was analyzed under the TEM microscope (Bonevich et al.,2010).

Fourier Transform Infrared Spectroscopy was done to examine the functional groups present and it was recorded using SHIMADZU- IR Affinity-1S FTIR spectrometer. For sample preparation of FTIR, solid KBr powder was allowed to dry for 2 h in a hot air oven. Then a small amount of the sample was ground with KBr and the pellet was prepared with the PCi Hydraulic Press dye. The pelleted sample was analyzed with an FTIR spectrometer between the range of 400-4000 cm^{-1} to obtain the corresponding peaks for the sample (Eid et al.,2022).

3.4. Characterization of Scaffolds

3.4.1. Morphological characterization of Ch/G, CN-Ch/G, nHAP/CN-Ch/G, NBG/TCP-Ch/G and NBG/GO-Ch/G scaffolds

For SEM analysis of all the scaffold samples, they were cut into small sections. Gold sputter coating was done on the sample's surface and then the sample was placed on aluminium stubs. The pore size of the control and composite scaffolds was calculated by using the ImageJ software. To prepare samples of cell-embedded scaffolds for SEM analysis, fixation and dehydration of cells were carried out with 1% glutaraldehyde solution and dehydration using a series of ethanol aqueous solutions. The ethanol concentration was consecutively increased from 30% to 70% and finally to 99.99% weight (Seda Tığlı et al.,2007).

3.4.2. Structural and Functional Characterization Ch/G, CN-Ch/G, nHAP/CN-Ch/G, NBG/GO-Ch/G and NBG/TCP-Ch/G scaffolds

XRD analysis of all the fabricated scaffolds was performed using Rigaku-MiniFlex 600 diffractometer that was operated at 40kV and 30mA with Cu Ka radiation (1.54Å) as a source. The samples were firstly crushed into powder using liquid nitrogen and then the above procedure was applied to obtain the corresponding peaks. The sampling time was 0.5s and the measurement temperature was 25°C. The scanning range of 2θ was between 20° C and 90°C (Peter et al.,2010).

The nature of interactions among the functional groups of the prepared scaffolds and their interaction with different nanoparticles were determined using Fourier transforms infrared (FTIR) spectroscopic analysis (FTIR, SHIMADZU-IR Affinity-1S FTIR) operated in the transmittance mode in 4500–450 cm^{-1} region. Hydraulic press was used to pelletize the scaffold specimens by mixing them with dry KBr powder (Peter et al.,2010).

3.4.3. Mechanical Strength analysis of all scaffolds

Mechanical strength for all the developed scaffolds was measured using a texture analyzer (CT3, Brookfield, USA). The height and diameter of the developed samples were noted down initially for all samples. Samples were sectioned into a cylindrical structure (10x10) mm^2. Samples were compressed to 50% of their existing height, using a 100N load along with a crosshead speed of $1mms^{-1}$. A stress-strain graph was also plotted using the texture analyzer software and compressive moduli were elucidated from the stress-strain plot's initial slope (Kim et al.,2010).

3.4.4. Swelling study of all scaffolds

Water uptake capacity for all the developed scaffolds was examined by dipping the dried scaffolds with a specific weight (W_d) in PBS for 24h at 37°C (Basu et al.,2020). The samples were removed from PBS solution at a specific interval of time (2,4,8,16 and 24 h) and then dried with blotting paper to obtain wet weight (W_t) for the test scaffold. Water uptake capacity for each sample was estimated by applying Equation 2.

$$Water\ uptake\ ratio = \frac{W_t - W_d}{W_t} \times 100 \text{--} Eq.2$$

3.4.5. *In-vitro* Degradation study of all scaffolds

In-vitro degradation of all the fabricated nanocomposite scaffolds was determined with respect to loss in mass (Wang and Sai et.al.,2013). This was done *in-vitro* with lysozyme (104 U/µg) solution prepared in PBS pH 7.4 at rt for the next 28 days. The scaffolds were cut into 2x2 cm^2 sections and their initial weight (W_i) was measured thereafter they were dipped in lysozyme solution for 4 weeks at rt. The media was replaced every three to four days throughout the experiment. The samples were removed at regular time intervals of 7 days, 14 days, 21 days and 28 days consequently. They were further dried in a vacuum oven at 40°C to measure the final weights (W_f). The weight loss (W_i-W_f) was plotted for all the scaffolds with respect to different time durations. The degradation percentage was calculated using Equation 3.

$$Degradation\ \% = \frac{(Wi-Wf)}{Wi} \times 100 \text{--} Eq.3$$

3.4.6. Porosity Measurement of all scaffolds

The liquid displacement method was used to determine the porosity of all the fabricated scaffold samples with and without nanoparticles (Guan et al.,2005). Absolute ethanol of known volume (V_1) with density ρ was used as displacement liquid because it can easily penetrate the

scaffolds and would not induce shrinking or swelling as a non-solvent of chitosan and gelatin. The scaffold samples of 2cm x 2cm were dipped in ethanol for 5 minutes and then the ethanol-impregnated scaffold's weight (V_2) was measured. After that scaffolds were removed and the residual ethanol volume was also recorded (V_3). With the above information, the porosity of scaffolds was calculated via the following equation:

$$Porosity\ \% = \frac{V_1 - V_3}{V_2 - V_3} \times 100 \quad \text{------Eq.4}$$

3.4.7. Thermal Stability Analysis (DSC)

The thermal degradation of all the developed scaffolds was investigated using Differential Scanning Colorimetry (DSC) (60 Plus, Shimadzu, Asia Pacific Pte Ltd). To determine the thermal stability of these samples, firstly they were crushed into fine powder. Then under a nitrogen atmosphere and a flow rate of 100mL/min and a temperature range of 20 to 400°C at -10°C/min, this study was conducted. The equipment was calibrated using indium and a pan which was used as a reference and a 5mg sample was used for this study (Jang et al.,2003).

3.5. *In-vitro* Cell culture study for developed scaffolds

3.5.1. Cell Culture

MG-63 cell line that was purchased from NCCS Pune India was used for all the cell culture experiments. For culturing of MG-63 cells, DMEM medium was used along with 10% FBS and 1% antibiotic solution. 1% sodium pyruvate was also used in 100mL T-flasks at rt with 5% CO_2 in a humid microenvironment. The media was changed every 3-4 days and cells were checked properly. For subculturing of MG-63 cells, firstly they were rinsed with PBS followed by detachment with trypsin- EDTA treatment (Tseng et al.2021). Fig.3.6 shows the isolated cells which will be utilized for further studies.

3.5.2. Cell seeding and culture in all the scaffolds

Scaffolds were sterilized using 70% ethanol from Merck followed by UV sterilization for 30 min thereafter they were placed on a 24-well plate. For the cell seeding experiment, the test scaffolds were incubated with 100µl osteogenic media for 20 min in a humid microenvironment. After which the media was discarded before seeding the cells. Cells were again detached with trypsin-EDTA treatment after the cells achieved 70-80% confluency. Then the cells were centrifuged at 300g at a temp. of 25°C for about 10 min and this pellet was further resuspended in the medium for cell-seeding over the wet scaffolds. Then 10^5 cells/cm^2

were taken for seeding onto each scaffold. The cells were pipetted over the media treated well plate which served as a positive control. 1.5mL of complete media was further incorporated into each well followed by incubation for predetermined periods. The media was replaced every three and four days (Bhisham et.al.,2019). Cells were cultured and they were retrieved every 7th, 14th and 21st day for *in-vitro* cell studies.

Figure 3.6: Cell isolated for in-vitro cell culture study

3.5.3. Cell morphology analysis via nuclear staining (DAPI)

DAPI (1 µg/mL) was used to stain MG-63 cell nuclei for visualizing osteoblast cell morphology and viability in the scaffold. The blue colour was observed when the dye was bound with the Adenine Thymine-rich clusters of the dsDNA. For this study, the media was removed from the culture plates and the samples were washed with PBS. Thereafter the cells were fixed with 4% paraformaldehyde and were allowed to fix over the scaffolds for 30 min at rt. Washing was performed after that and then they were incubated with 0.1% (v/v) triton X-100 for the next 10 min. 1% BSA was added to the cells to stop the reaction and to avoid the non-specific binding of the stain and it was further incubated for 30 min. The unbound BSA was again washed with PBS. Finally, the osteoblast cell-seeded scaffolds were incubated with DAPI for 30 min in a dark environment to allow the absorption of the stain by the cells. Final washing with PBS was performed. Followed by fixing the cells with 80% glycerol to prevent the moisture from evaporating (Tarnowski et al.,1991). The cell-loaded scaffolds were visualized under a fluorescence microscope (BM-LCD802, LEEDZ MICRO IMAGING LTD. UK) to view stained nuclei (Sahi, et al.,2021).

3.5.4. Cell viability analysis

MTT assay

Cell viability and functional activity were calculated using 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) assay, which measures the mitochondrial dehydrogenase activity of viable cells spectrophotometrically. Cell-seeded scaffolds were incubated in a humidified environment at 37°C under 5% CO_2 for the 3rd, 5th and 7th days respectively. Briefly, 20µl of MTT solution (5mg/mL) was added to the cell suspension (96 well plate) followed by incubation for 4 h. After that, the whole medium was taken out and the wells were substituted with 200µl of DMSO and again kept in an incubator for 4 h. Thereafter the absorbance was recorded by using a microplate reader (2030 multilabel reader victor X3, Perkin Elmer, USA) at a wavelength of 595nm (Kumar et al.2018).

Live-Dead assay

Dual fluorescent staining (DFS) was done using calcein acetoxymethyl (CAM) ester and ethidium homodimer-1(EHD) to evaluate cell viability in the scaffolds. It labels the intracellular esterase activity and plasma membrane integrity respectively giving green and red fluorescence of calcein and EHD which is easily detectable in the fluorescence microscope (Raucci et. al.,2019). The solution of EHD (6µM) and CAM (1µM) was mixed with cell suspensions to stain the cells in scaffolds (Elieh-Ali-Komi et al.,2016).

3.5.5. Osteogenic Differentiation Potential

Alkaline phosphatase assay (ALP)

Osteogenic differentiation of MG-63 cells was estimated by the Alkaline phosphatase activity of the cells. It is considered to be the most promising early differentiation marker for bone cells (WB et. al.,1990). For this study, 200µl of 1 mg/mL p-NPP tablets were taken as a substrate for osteoblast cells (Sabokbar et al.,1994). Thereafter ascorbic acid (2 µg/mL) and ß-glycerophosphate (10mM) were added to the osteogenic media. Then after a fixed duration of culturing (7 days, 14 days, 21 days), the media was removed from the cell-scaffold construct present in the wells. It was followed by washing scaffolds with PBS. These cell-seeded constructs were treated with 50 µL lysis (0.5% Triton-X 100) solution and then incubated with p-NPP solution concentration as mentioned above for 2h at rt. In this reaction, p-

nitrophenylphosphate gets hydrolysed to p-nitrophenol by the action of ALP (WB et al.,1990). The reaction was stopped by adding 100µL (0.2M) NaOH solution. Finally, the OD was recorded at 405 nm with UV-Vis spectrophotometry (Sabokbar et al.,1994).

Alizarin Red staining assay (ARS)

Alizarin red staining was performed for MG-63 cells over fabricated scaffolds with nanohydroxyapatite and copper nanoparticles to determine the calcium deposition in the cultured tissue constructs. The samples were first washed and then fixed with 2.5% glutaraldehyde. The fixed samples were further washed with PBS and then incubated with 1mL ARS solution for 1h at 37ºC. The ARS solution was prepared by dissolving 0.5g of ARS into 25 mL DDW at a pH between 4.1 to 4.3. After 1h incubation, the samples were again washed and visualized under a fluorescent microscope and the images were obtained. The further quantification of calcium deposition was performed using cetylpyridinium chloride (CPC) for which the ARS-stained samples were incubated again for 1h in 500µl CPC solution. During ARS staining, the calcium ions in the sample form complexes with the ARS dye, producing a red colour indication of calcium deposits. Therefore, CPC is employed to selectively elute this calcium-bound dye complex, allowing for the quantitative analysis of calcium content. Finally, the samples were removed from the solution and the absorbance was recorded at 550nm using a UV spectrophotometer (Gregory et al.,2004; Bhisham et al.,2019).

3.5.6. Statistical analysis

All the experiments in this study were conducted in triplicates and all the results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was carried out for the statistical analysis of the experiments with Tukey's multiple comparison tests. For two sets of data when p was less than 0.05 ($p<0.05$), it was regarded as statistically different. OriginPro 2020 was utilized to plot all the graphs and the background noise in the fluorescent microscopic images was removed through Image J software.