Chapter 3- Material and methodology

3.1 Material

Collagen peptide (bovine origin), chitosan (medium molecular weight), ciprofloxacin hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Lysozyme, and Trypsin (0.25%) were purchased from Himedia, India. Paraformaldehyde, Triton X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT), ammonium hydroxide, glacial acetic acid (100%), chloroform, ethanol, dimethyl sulfoxide (DMSO), nitric acid, 4,6-diamidino-2-phenyindole (DAPI), PCL (MW-80000), Calcium Peroxide (CPO) and hematoxylineosin staining solution were purchased from Sigma Aldrich, (St.Louis, USA). Glutaraldehyde and catalase were purchased from SRL chemicals. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and antibiotic–antimycotic solution were purchased from Gibco, (USA). Phosphate buffer solution (PBS) was purchased from Invitrogen, USA, and Cyclohexane (Merk Millipore). Double distilled water (DDW) was utilized throughout the experiment.

3.2. Method of scaffold preparation

3.2.1. Fabrication of ciprofloxacin loaded collagen-chitosan scaffold

Porous biopolymeric ciprofloxacin loaded collagen/chitosan scaffold (COL/CH/CPX) was prepared through freeze drying process. Collagen (1.5 w/v) and chitosan (1.0% w/v, 1.5%w/v and 2.0w/v %) solution were prepared in 1% glacial acetic acid solution. Ciprofloxacin was added in during the preparation of collagen/chitosan blend solutions to obtain antibiotic concentration 2mg/ml. The collagen/chitosan (COL/CH) scaffolds were fabricated through blending of COL/CH solutions in different concentration as mentioned in the table 3.1

	Collagen (%)	Chitosan (%)	Result
Scaffold-1(SC-1)	1.5	1.0	Scaffold prepared
Scaffold-2(SC-2)	1.5	1.5	Scaffold prepared
Scaffold-3(SC-3)	1.5	2.0	Scaffold prepared

Table 3.1. Details of scaffold preparation at varied concentration of collagen and chitosan solutions.

After appropriate mixing of COL/CH solution in above mentioned concentrations the obtained solutions were placed at -20 °C for overnight. After freezing, the frozen sample was subjected to lyophilisation for 24 h using lyophilizer (Lab Corno USA). Crosslinking is necessary to maintain the physical and chemical structure of scaffold at site of application.

Dual cross linking was performed to provide the better mechanical strength to scaffold. Glutaraldehyde as a cross linking agent provides good mechanical strength but makes the scaffold brittle. Besides glutaraldehyde, EDC/NHC was also recruited as a cross linking agent but it also failed to maintain the structural integrity of scaffold. Therefore, dual cross liking has been employed to attain the scaffold having structural integrity and fidelity. By using varied concentrations of glutaraldehyde and EDC/NHC at different time points the optimum concentration was achieved to attain the best scaffold. In the first step, scaffold was cross-linked using, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) for 12 h. The cross-linking solution was prepared as 3 % EDC/NHS [2:1 (w/w] in [(95:5 v/v (ethanol: water)]. After 12 h of treatment, scaffold was transferred in a second cross-linking solution containing 1% glutaraldehyde in 50:50 v/v (ethanol: water) for 12 h. After cross-linking, the obtained scaffolds were washed three times with double distilled water. The cross-linked scaffold was freezed overnight and then freeze-dried using lyophilizer (Lab Corno USA) for 24 h.

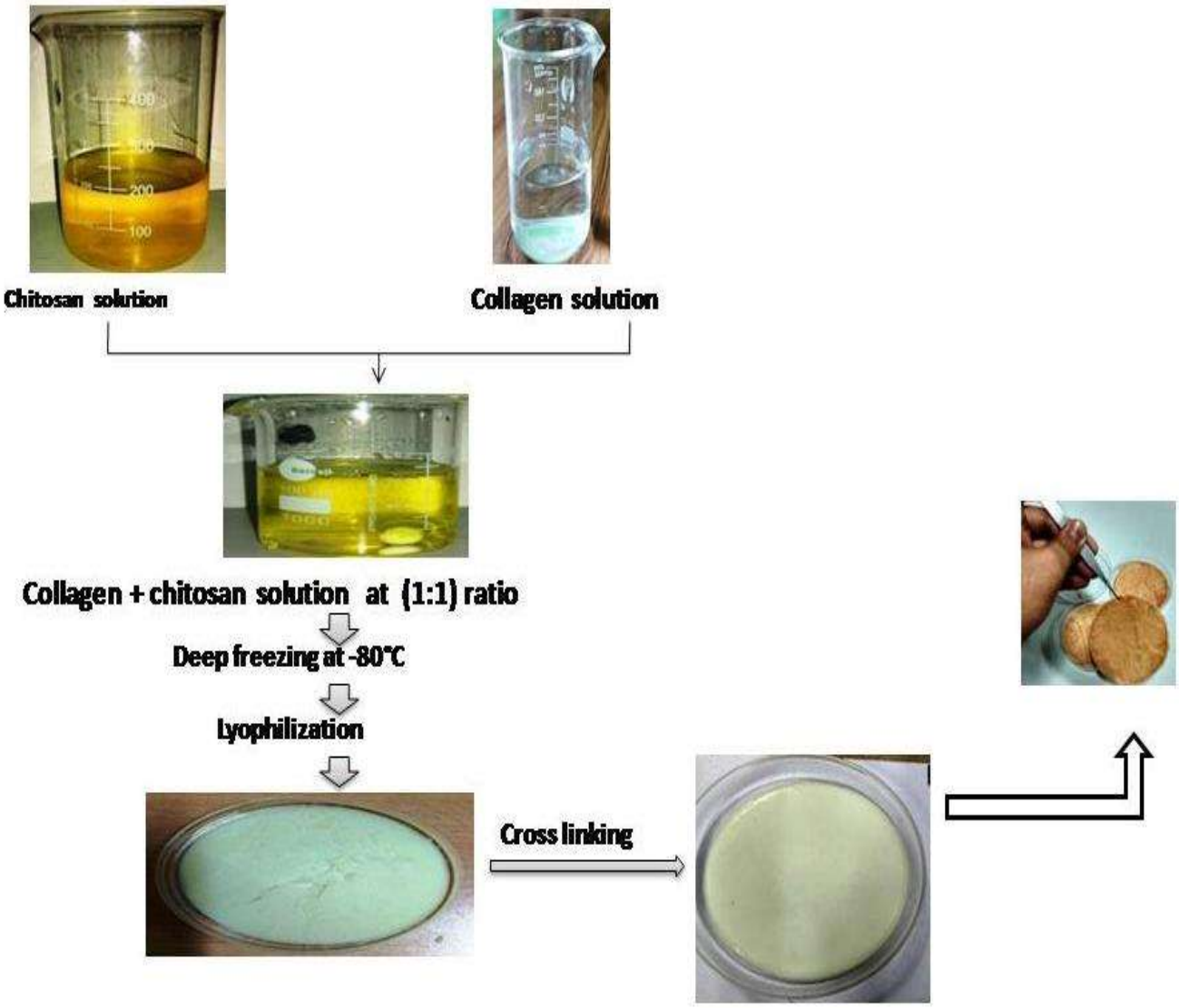

Figure 3.1. Fabrication of oxygen producing ciprofloxacin loaded collagen-chitosan scaffold

3.2.2. Fabrication of oxygenating ciprofloxacin loaded collagen-chitosan scaffold

The collagen/chitosan scaffolds were fabricated using a freeze-drying method using collagen and chitosan solution as shown in figure 3.1. Two different solutions of chitosan (1.5% w/v) and collagen (1.5% w/v) were prepared by dissolving in 1% and 2% acetic acid solution and the mixed in equal ratio. Ciprofloxacin hydrochloride was mixed in the solution to achieve the antibiotic concentration of 1 mg ml^{-1} in solution. After mixing the solution in the above motioned concentrations it was placed at −80 ◦C for overnight freezing. Afterwards, the frozen samples were lyophilized for 24 h using lyophilizer (Labcono USA). The obtained scaffolds were cross-linked in two steps. In the initial step, the scaffolds underwent the process of cross-linking using, EDC and NHS for 12 h. The cross-linking solution was prepared by 3% EDC/NHS [2:1 (w/w) in (95:5 v/v (ethanol:water))]. In the later step the scaffolds were transferred in a second cross-linking solution containing 1% glutaraldehyde in 50:50 v/v (ethanol:water) for 12 h. Subsequently, cross-linking scaffolds were washed three times with DDW insure the removal of residual of EDC/NHC and glutaraldehyde. Cross-linked scaffolds were freezed overnight and then subjected to freeze drying using lyophilizer (Lab Corno USA) for 24 h[230].

3.2.2.1 PCL-CPO hybrid coating

PCL pellets were dissolved in chloroform to make 1% (w/v) solution. Catalase enzyme was mixed in PCL solution to make the concentration of enzyme equal to 500 U ml−1. CPO powder was added in chloroform to make four different solutions ranging from 1% to 4%, under constant stirring until a homogeneous solution was obtained. Finally, the scaffold was dipped into PCL solutions for 10 min under constant stirring. Depending on the coating of CPO of 1%, 2%, 3% and 4% the scaffolds are denoted as SC-1, SC-2, SC-3 and SC-4

respectively. SC-0 was devoid of PCL and CPO coating. The scaffolds were vacuum dried and stored.

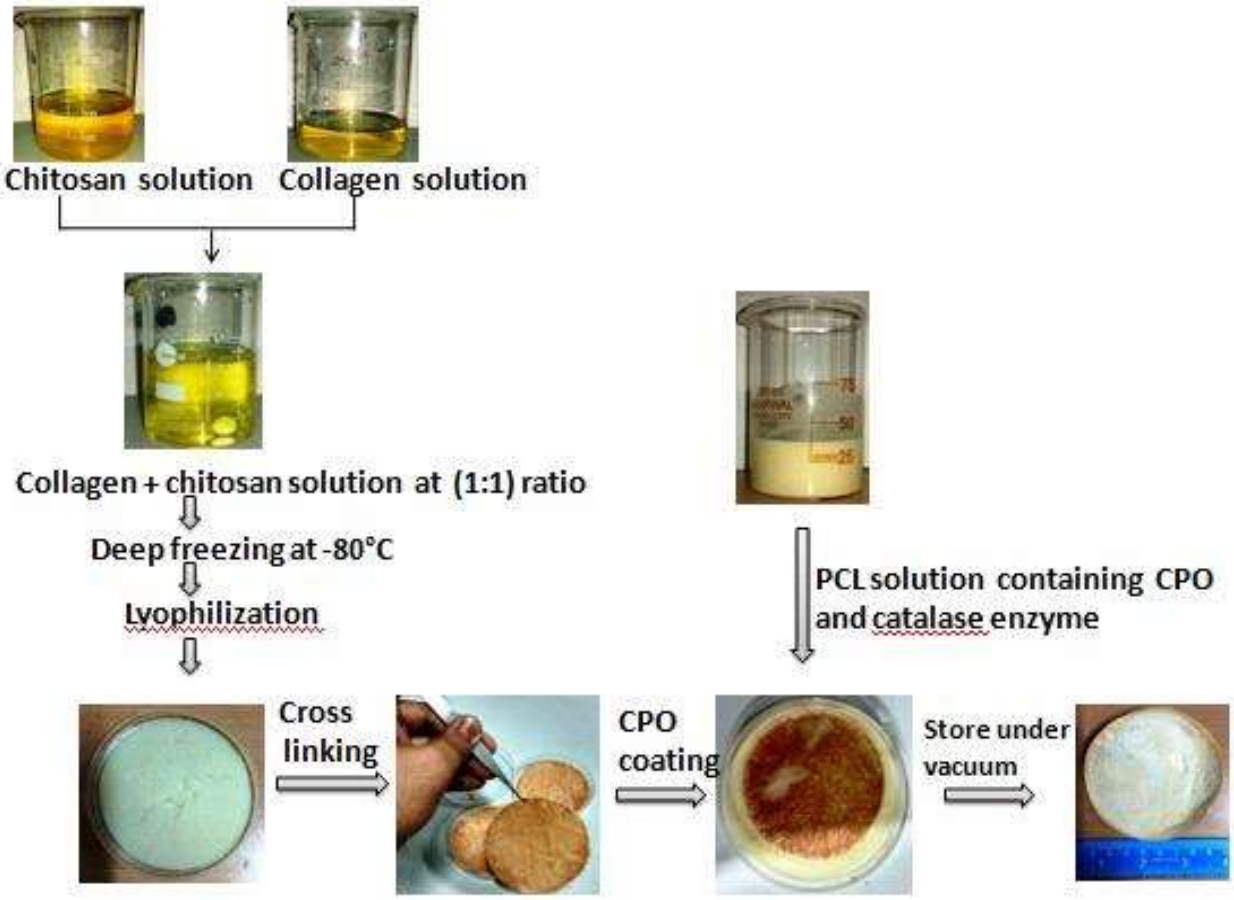

Figure 3.2. Schematic diagram of scaffold preparation.

3.3. Characterization of scaffold

3.3.1. Physical and chemical characterization

3.3.1.1. Morphological analysis

The developed scaffold was characterized for its morphology and pore size. The moisture present in the scaffolds was removed using vacuum dryer at 40 °C for the duration of 3 h. Each Scaffold having a dimension of 5 × 5 × 1 mm (L × B × H) was coated with gold sputter coating to make them conductive. Scanning electron microscope (SEM) (Zeiss Evo-18, SEM, Germany) was used to study the microstructures of the scaffolds and the micrographs obtained were used for microstructure and pore size determination by ImageJ software (NIH, USA). Average pore size was calculated using ten micrograph images. SEM image of each oxygenating scaffolds was analysed for elemental mapping with Energy Dispersive X-ray (EDX) spectrometer equipped with SEM. EDX was used to analyse the presence of CPO coating in different scaffolds[231].

Porosity is an important parameter in reference to cell holding capacity of scaffold. Porosity of the developed scaffolds was measured using the liquid displacement method. In this method, the scaffold of weight W was immersed into a graduated cylinder containing a known volume (V 1) of ethanol.

The cylinder was placed in a vacuum to force the ethanol into the pores of the scaffold until no air bubbles were observed. The total volume of the ethanol having scaffold was recorded as (V 2). The scaffolds were removed from the cylinder and residual volume of ethanol in scaffold was measured and recorded as V 3.

Experiment was performed in triplicate and average value was taken. The porosity of scaffold was measured by the following formula[232]:

$$Porosity = \frac{V_1 - V_3}{V_2 - V_3} \times 100$$

3.3.1.2. Swelling percentage

Conventional gravimetric method was applied for the estimation of swelling percentage. Swelling behavior of scaffold was evaluated by cutting the scaffold into pieces having dimension of 15 mm ×15 mm × 5 mm (length× weight × height) and weight was measured. Scaffolds were immersed into a beaker containing Phosphate buffer saline (PBS) of pH 7.4 at a temperature of 37°C. Afterwards, scaffolds were taken out of PBS, wiped with filter paper and weighed at the interval of one hour for the period of 24 hrs. Each experiment was run in triplicate and the swelling percentages was calculated as-

$$Swelling\ Percentage = \frac{W_t - W_o}{W_o} \times 100$$

Where W_0 is the initial weight of the scaffold, $W_t$is the weight of scaffold after different time intervals[136].

3.3.1.3. Contact angle measurement

Contact angle analysis was carried out to find out the wettability of the developed scaffold. The contact angle of developed scaffolds were measured at room temperature (25 ± 1°C) using sessile drop method by placing 10 µL of distilled water over the surface of develop scaffolds and angle was measured using Image J. software. Contact angle was measured by taking six reading at different parts of the scaffold and value was averaged[233].

3.3.1.4. Biodegradation

An ideal scaffold should possess the degradation behavior to cope with the process of regeneration of damaged tissues and organs. Hence, biodegradation behaviour of scaffold was evaluated by measuring the change in weight of scaffold in different time intervals in phosphate buffer saline (PBS) at pH 7.4. The scaffolds were sterilized by immersing in 70% ethanol, and weight was recorded. Lysozyme degradation method was adopted to evaluate the degradation behavior of scaffolds .Degradation study was performed in 0.05 M PBS (pH 7.4) at 37°C containing 1.6 µg/ml (112 Units/ml) lysozyme (hen egg-white). The lysozyme solution was renewed in every three days. Scaffolds were washed with water, dried, and weighed for the period of 28 days at the interval of seven days. Percentage of weight remained was calculated using the following equation[234]:

$$Weight\ remained = \frac{W_0 - W_t}{W_o} \times 100$$

Where W_0 is the dry weight of scaffold before degradation and W_t is the dry weight of scaffold after degradation.

3.3.1.5. Water Vapor Transmission Rate (WVTR)

The moisture permeability of the developed scaffolds was determined by measuring the water vapor transmission rate (WVTR) across the scaffolds as stipulated by the ASTM E 96/E96M-05 standard[235]. According to this protocol a glass tube with internal diameter of 2 cm and height 1.6 cm was filled with 4 gm of deionized water. Scaffolds were dried using hot air oven to remove any moisture present in scaffolds. Scaffolds were fixed at the top of the tube. Sealant was used to prevent any loss of water vapor from the periphery of the tube.

Tubes were placed in oven having relative humidity 50% and temperature 37^0 C. After 24 h, the weight of the water remaining in the tube was measured. Total number of samples taken is three (n=3).

$$WVTR = \frac{4 - Weight\ of\ second\ day}{area\ of\ scaffold\ X\ 24}$$

WVTR was calculated in (g/m^2 /h).

3.3.1.6. Mechanical study

The mechanical properties of the scaffolds were determined in terms of tensile strength using texture analyzer (CT3Brookfield, Germany). The scaffolds were cut into quadrangles of 5 cm×1 cm, and the thickness of the scaffold was maintained around 3 mm, which was measured by vernier caliper (Mitutoyo 8" Vernier Caliper Model 502-231). Tensile strength of scaffold was measured in both dry and wet condition. The test speed was maintained around 0.05 mm/s and the gripping distance was 40 mm. All the experiments were performed in triplicate (n=3).

3.3.1.7. FTIR study

Fourier transform infrared spectroscopy (FTIR) spectroscopy is the one of best method to characterize functional groups, bonding types and nature of compounds. The technique helps to explore the functional groups and the chemical bonds present in the scaffold. This technique is very useful to determine the primary chemical structure and composition of chemical compound. The chemical structure of developed scaffolds was analyzed using FTIR by KBr pellet method (FTIR-8400S, Shimadzu, Japan).The scaffolds (0.3-0.5 mg) were

ground in about 80 mg of spectral-grade potassium bromide and pressed into pellets under about 5–6 tons/cm^2 pressure. The analysis was done in the range of 4000 to 600 cm^{-1}.

3.3.1.8. XRD analysis

X-ray diffraction analysis was carried out to investigate the crystallinity of the scaffold prepared. XRD is used to identify the drugs present inside the scaffold as each of these drugs has a unique X-Ray Diffraction (XRD) pattern that makes their identification possible. Intensity of XRD peak also used to correlate the relative quantity of a particular material in different samples.The scaffolds were characterized by using automated XRD (Rigaku-Ultima IV, X-ray diffractometer, USA) using Cu-Kα radiation. The samples were scanned in the 2θ range of 10° to 70° at a scan rate of 2θ=10/min. and the X-ray source was operated at 30 kV and 30 mA.

3.3.1.9. Thermal property study

Thermal gravimetric analysis (TGA) is a method of thermal analysis in which the mass of a sample is measured over time as the temperature changes, therefore a Pyrolytic pattern of the samples was obtained using a Thermo Gravimetric Analyzer (TGA-Q50) under N_2 atmosphere at a heating rate of 10°C/min with a sample size of 5 mg. TGA provides information about the thermal stability of the material phase transitions, adsorption and desorption oxidation, combustion and thermogravimetric kinetics. TGA study defines the applicability of polymeric material in tissue engineering as the material used is suitable for the autoclave or it get decomposed during the process of sterilization. Chemical changes occur during the process of heating. DSC is used to evaluate the thermal transition of polymeric materials which includes the glass transition temperature

(T_g), crystallization temperature (T_c), and melting temperature (T_m). DSC is used to measure the stability of polymer by obtaining Gibbs Free Energy at any given temperature. These parameters were obtained using Differential Scanning Calorimeter (DSC Q200) under N_2 atmosphere at a heating rate of 10°C/min with the sample size of 5 mg.

3.3.1.10. Oxygen releasing behavior

The oxygen release profiles of developed scaffolds were measured by measuring the dissolved oxygen in deionized water. Dissolved oxygen (DO) concentration was recorded using a dissolve oxygen probe attached with dissolve oxygen meter (Orion Star™ A216 pH/Dissolved Oxygen Bench top Multiparameter Meter). DO probe was attached to the borosilicate vial to allow the measurement of dissolve oxygen without interference from oxygen environment. Scaffold of equal size having dimensions of (2 × 2 cm) were cut and placed inside the 10 ml of deionisedwater. All measurements were carried out under a bio safety cabinet and oxygen concentration was measured at the interval of 24 hrs from day one to tenth day. All measurements were performed in triplicate. Deoxygenated media was taken as the control for the measurement.

3.3.1.11. Drug release study

Tissue engineering employs biomimetic materials that create a suitable microenvironment for adhesion, migration, proliferation of differentiation of cells. These processes can be positively facilitated by the addition of drugs that have an influence on cellular function and tissue regeneration. Tissue engineering employs one or more of these three integral components: biomaterial matrices, living cells, and bioactive drugs. Different signaling processes are involved in the process of wound healing. Therefore various signaling

molecule such as peptides, proteins, growth factors, cytokines, and other bioactive molecules are used as drug which leads to the faster wound recovery. Integration of drug in biomaterial has increasingly become the integral component and hence need for more controlled release of drugs is needed to ensure proper and integrated signaling during the wound healing will lead to quantity and quality of tissue regeneration.

Controlled drug delivery can be accomplished by physically or chemically adsorbing the drug onto the surface1 of scaffold or encapsulating the drug within scaffold, or by incorporating drug delivery systems on the scaffold. The subsequent release of the drug occurs by diffusion or due to degradation of the scaffold or encapsulating material. The quantity and duration of drug released can be controlled by altering the composition of the material, the delivery system, or the methods of drug integration.

Drug release study of ciprofloxacin form collagen-chitosan scaffold was carried out using an automated Franz diffusion cell apparatus (Microette Plus™; Hanson Research, Chatsworth, USA) with the basket method at 100rpm. A volume of 900 mL phosphate buffer pH 7.4 equilibrated at 37°C was utilized as dissolution fluid. The prepared scaffolds were placed between the donor and receptor compartments. Isotonic phosphate buffered saline (pH 7.4) was used as a release medium in the receptor chamber and was maintained at 37 °C with stirring rate of 100 rpm. Aliquot samples (2 ml) were withdrawn by the auto sampler and appropriately diluted to analyze for drug content spectrophotometrically at 540 nm (Shimadzu, UV-1800 spectrophotometer, Japan). A cumulative percentage of release of ciprofloxacin form scaffold was *in vitro* examined for a period of 20 h, and a relationship between the percentage drug release and releasing time was plotted [138]. The obtained data

were fitted on the Korsmeyer-Pappas model and used to determine the type of diffusion during the drug releases.

3.3.1.12. Hemocompatibility study

Hemocompatibility test of the developed scaffolds was performed to measure the extent of haemolysis of RBCs during wound healing as per ASTM protocol. Fresh goat blood was collected in the presence of Trisodium citrate (TSC) (3.8 gm in 100 ml of blood). Thereafter, citrate blood was diluted with normal saline in 8:10 ratio using 0.5 ml of diluted blood mixed with 9.5 ml normal saline in a falcon tube. The developed scaffolds having dimension of 10 mm ×10mm×3mm (Length × Breadth × Height) was put inside the falcon tube at 37°C at 50 rpm. Two types of controls were taken, namely positive and negative control. In case of positive control, the mixture of diluted blood (0.5 ml) and 0.01N HCl (0.5ml) was mixed and volume was made 10 ml with normal saline. In case of negative control, 0.5 ml of diluted blood was mixed with normal saline to make volume up to 10ml. All the samples were centrifuged, and the absorbance of the supernatant was measured at 545 nm to calculate the percent of haemolysis. The experiment was run in triplicate and the results were evaluated based on the following parameters-

$$\%\, Hemolysis = \frac{OD_{test} - OD_{negative}}{OD_{positive} - OD_{negative}} \times 100$$

If total haemolysis is more than 20 % then the developed scaffold is non-hemocompatible, if less than 5% then highly hemocompatible, and If 5-10 % then hemocompatible[37] .

3.3.2. In vitro cell culture study

3.3.2.1. Cell isolation and cell attachment study

Fibroblasts were isolated from human skin. Skin was washed several times with phosphate buffer saline (pH-7.4) supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml). Fat layer was removed using scalpel as fat layer hinders in enzyme digestion processes. Trypsin (0.25%) digestion was applied for 30 min to separate the epidermis from dermis. Epidermis was peeled out gently from the dermis using forceps. Dermis was washed again using PBS and digested using collagenase type-1 in DMEM medium supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) inside the incubator at 37°C, 5% CO_2) for three hrs. After digestion, media was centrifuged at 300g for 10 min. and supernatant was discarded while cell pellet was cultured with complete media (DMEM with 10% FBS supplemented with penicillin (100 U/ml), streptomycin (100 U/ml)) in cell culture flask inside the CO_2 incubator at 37°C, 5% CO_2, and 95% humidity. Culture media was changed at every three days. Cells were passed at confluence and after 4-5 passage fibroblast was used for further studies.

Scaffolds were sterilized using ethanol and UV light before cell seeding. Trypsinization was carried out using 0.25% trypsin solution. Scaffolds were homogenously seeded with fibroblasts. Scaffolds were seeded with 1 ml of seeding solution containing 1.25×10^5 fibroblast in each scaffold. After cell seeding the fibroblast cells were allowed to settle and attach inside the scaffolds. Culture media was added, and cell seeded scaffold was placed in tissue culture plate with complete culture media for cell growth inside the incubator at 37°C, 5% CO_2, and 95% humidity with 1% oxygen to induce hypoxic condition. The fibroblast morphology and attachment over cultured scaffolds were studied for the period of 7- and 14-

days using scanning electron microscopy and florescence microscopy. Briefly, Attachment of fibroblasts on the scaffolds was qualitatively evaluated by scanning electron microscope (SEM). The Scaffolds were seeded with fibroblast and cultured for 7 and 14 days in a CO_2 incubator. The scaffolds were then washed with PBS three times and then fixed with 2.5% glutaraldehyde. The scaffolds were further serially dehydrated using ethanol solutions (10, 20, 30, 50, 70, and 100 %) followed by air drying and gold coating and observed under SEM [38](Zeiss Evo-18, SEM, Germany). The proliferation of cells in scaffolds was studied using fluorescentstain DAPI and images were collected using fluorescence microscope.Survivability of fibroblast was evaluated after 7 and 14 days using a live/dead cell assay. Viability of fibroblast over scaffold was assessed by using 5 μMCalcein AM and 3 μM Ethidium homodimer-1 solutions. Cell seeded scaffolds after stipulated time was washed with PBS and then incubated for 30 – 40 min. in staining solution. Stained samples were then washed with PBS and images were taken using fluorescence microscopy (Olympus BX51, Japan)[16].

3.3.2.2. MTT Assay

MTT assay is a colorimetric assay used to evaluate the cellular metabolic activity as an indicator of cell viability. Metabolic activity of fibroblast over developed scaffolds was examined using the MTT (3- dimethylthiazol-2, 5-diphenyltetrazolium bromide) as a substrate[39–41].Fibroblast cells were cultured over the developed scaffold up to 14 days and metabolic activity of cells was examined on 7^{th} and 14^{th} day[42,43]. After the stipulated time the scaffolds were washed with PBS twice followed by addition of MTT (10μLof a 5 mg/ml in PBS) in the wells of culture plate. Afterwards, the cells were incubated at 37 °C for 3-4 hrs in a CO_2 incubator. After the stipulated time the medium was discarded and the 200 μL of

DMSO was added in each well to solubilise the formed formazan crystals and the absorbance was read at the wavelength of 540 nm using the Micro plate reader ((MK3, Thermo Electron Corporation, UK).

3.3.2.3. DNA Content estimation

Proliferation of fibroblast over developed scaffolds was evaluated through total DNA content assay. The amount of DNA associated with the fibroblast over the developed scaffold was quantified with a PicoGreen Quantification Kit (Molecular Probes). Briefly, the cultured scaffolds were taken out at different time intervals (7^{th} and 14^{th} day), washed thrice with PBS and treated with 1.0 ml of cell lysis buffer for 30 min. Lysis buffer contains 10 mM Tris (pH 8), 1mM EDTA, and 0.2% (v/v) Triton X-100. After cell lysis the samples were centrifuged at 300g for 10 minute and supernatant was collected while pellet was discarded. 100µl of sample was mixed with 100 µl of DNA binding fluorescent dye solution. Intensity was measured through a spectrofluorometer at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Standard curve was prepared using lambda DNA. All the experiments were run in triplicate[44].

3.3.3. Antibacterial test

Infections in surgical site poses significant clinical problem during surgery and subsequent wound healing. These infections can prolongs the recovery period, implant failure and increase cost. Therefore to prevent the infection in scaffold, the scaffold should have antibacterial property. Therefore during the fabrication of scaffold, antimicrobial agents such as antibiotics are added so that scaffold can contain the innate antibacterial properties. Antibacterial properties of scaffold were evaluated using agar diffusion method. Antibacterial test of developed scaffold was performed, using zone of inhibition method

against the *E. coli* and *S. aureus* which are most common bacteria present in wound site. Sterile Lenox broth (LB) media was used for the bacterial inoculation and inoculated plates were employed for antibacterial test. Scaffolds were cut in circulator shape (1.0 cm in diameter) and placed gently over the media and incubated at 37ºC for 24 hrs. Antibacterial activity of control and SC-4 was measured by measuring the zone of inhibition. In control scaffold without antibiotic was used. The study was performed in triplicates (n=3).

3.3.4. *In vivo* and histological study

3.3.4.1. *In vivo* study

All the experiment on animals were conducted in accordance with the ethical principles adopted by the committee for the purpose for control and supervision of experimental animals (CPCSEA), Government of India and were approved by the Institutional Animal Ethical Committee, Institute of Medical sciences, Banaras Hindu University. Skin flap model was used to evaluate the effect of scaffolds in wound healing study. Two months old albino male rats weighed (30 ± 5 g) and housed in polypropylene cages in a well-ventilated room exposed to ambient conditions of 12h photoperiod, 25 °C temperature with a relative humidity of 50%. The rats were fed with food and water *ad libitum*. Before the implant, the vasculature pedicles were trimmed. The full thickness of the dermis having dimension of 1.5 cm ×1.5 cm, was removed from the back of rat. Wound in rats were created after anesthetizing them with ether and oxygen-releasing scaffold was implanted on the surface of wound. Rats were housed singly in separate cage and wound was inspected each day until the wounds healed completely. Rats without scaffold were used as control. The study was performed in triplicate. The degree of wound healing was determined by measuring the area

of the wound with respect to a ruler by means of Image J software. Wound healing rate was calculated using the formula available in literature [45].

$$\text{Wound healing rate} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 represents the initial area of wound, $A_t$represents area of wound remained after time(t).

3.3.4.2. Histological examination

Histological studies were executed using Haematoxylin and Eosin staining. For histological study the samples were excised from the area of the wound using a biopsy punch needle at different time intervals (5^{th}, 10^{th} and 15^{th}day). The biopsy sections were fixed in 10% formaldehyde solution for 24 hrs followed by serial dehydration with different grades of alcohol, cleared in xylene, and embedded in paraffin wax. These paraffin blocks of different samples were sectioned into 4μm thickness using microtome and mounted on poly-L-lysine coated slides .The sections were deparaffinised in xylene and rehydrated in different grades of alcohol followed by Haematoxylin-Eosin staining and observed under an inverted routine microscope (TS100, Nikon, Eclipse, Japan).

3.3.5. Statistical analysis-

The experiments were done in triplicate, and data were presented as mean ± SD. Statistical significance was determined by one way ANOVA. ‘p’ Value less than 0.05 was considered as significant.