2. EXPERIMENTAL PROCEDURES

This chapter describes different experimental techniques used for the synthesis of gelatin grafted polymers, fabrication of 3D scaffolds, characterization and their biological evaluation.

2.1. Chemicals and Materials

3,6-Dimethyl-1,4-dioxane-2,5-dione (D,L-Lactide), Tin(II) 2-ethylhexanoate, *N*-hydroxusuccinimide (NHS), bovine serum albumin (BSA) lyophilized powder, Thioflavin T, GPTMS, Hematoxylin- Eosin (H&E) solution and methylthiazolyldiphenyl-tetrazolium bromide salt (MTT) were purchased from Sigma-aldrich. Benzyl alcohol was purchased from Merck Pentaerythritol was purchased from SRL chem. lab. Gelatin Type A (from porcine skin), succinic anhydride, pyridine, and *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC.HCl) were purchased from Hi-Media Laboratories and used as received. Dialysis tubing having MWCO = 100000 was purchased from Cole Parmer. Analytical grade solvents like dichloromethane (DCM), tetrahydrofuran (THF), dimethylsulfoxide (DMSO), dimethylformamide (DMF), hydrochloric acid (HCl) were purchased from SRL chem. lab. HPLC grade DMF for the GPC mobile phase was purchased from Spectrochem (India).

2.2. Synthesis of gelatin grafted PDLLA (l-pLG and ss-pLG)

2.2.1. Synthesis of linear and star shaped poly(D,L-Lactide) (l-PDLLA and ss-PDLLA)

ss-PDLLA was synthesized by our previously reported protocol [62] with slight modification. In brief, D,L-lactide (8 g, 5.5×10^{-2} mol) and pentaerythritol (10.78 mg, 0.079×10^{-3} mol) were taken in a 100 mL dry Schlenk tube under inert nitrogen environment and dried under high vacuum at 80 °C for 2 h. Then, $Sn(Oct)_2$ (40µL, 0.5 % w/w to D,L-Lactide) were added to the Schlenk tube. The reaction mixtures were purged with nitrogen for 30 min and then Schlenk tube was tightly closed, and polymerization was carried out at 150 °C for 12 h. The polymerization reaction was

stopped by freezing the reaction mixture in liquid nitrogen. The crude polymer was dissolved in 10 mL of THF and precipitated out from hexane. Precipitation procedure for repeated twice and the polymer was dried under high vacuum at room temperature to get pure ss-PDLLA.

In case of l-PDLLA, 7.5 g of D,L-lactide (5.2×10^{-2} mol) monomer was taken along 7.8 µL of benzyl alcohol (81.12 mg, 7.5×10^{-5} mol) and 37.5 µL (0.5% w/w ratio to D,L-Lactide) of $Sn(Oct)_2$. The remaining experimental procedures were same as ss-PDLLA.

2.2.2. Synthesis of carboxyl terminated PDLLA (l-PDLLA-COOH and ss-PDLLA-COOH)

ss-PDLLA [4.0 g, 0.02×10^{-3} mol, calculated with respect to its M_n (NMR) = 186 300 g mol^{-1}] and succinic anhydride (40 mg, 0.4×10^{-3} mol) was taken in a two neck round bottom flask and dried completely under high vacuum. Reaction was started by adding 2 mL of pyridine to the 100 mL of DCM reaction mixture [63]. It was stirred for 48 h. After the reaction time, reaction mixture was washed with warm double distilled water (50 – 60 $^{\circ}$C) to remove the unreacted succininc anhydride. Then they were precipitated into excess methanol. The product was vacuum dried and kept in desiccator until further use.

For l-PDLLA-COOH, l-PDLLA [4.5 g, 0.022×10^{-3} mol, calculated with respect to its M_n (NMR) = 206 010 g mol^{-1}] and five folds higher succinic anhydride (11.07 mg, 0.11×10^{-3} mol) was taken in a flask along with pyridine. The reaction condition was same as for ss-PDLLA-COOH synthesis

2.2.3. Synthesis of block copolymer Gelatin grafted PDLLA (l-pLG and ss-pLG)

ss-PDLLA-COOH [1.9 g, 0.01 × 10^{-3} mol, calculated with respect to its M_n (NMR) =186 516 g mol^{-1}] and NHS (13.15 mg, 0.114 × 10^{-3} mol) was dissolved in DCM (25 mL) in the presence of EDC.HCl (43.7 mg, 0.228 × 10^{-3} mol). The reaction was stirred for 24 h. Then, DCM was evaporated by rotary evaporator and the activated end terminal –COOH was re-dissolved in acetonitrile and added into gelatin (4 g, 0.02 × 10^{-3} mol) solution in PBS of pH 8.0. The reaction mixture was stirred for 24 h and the crude product was dialysed against deionized water for 48 h at 40 °C. Water was changed every 6 h [2]. Then the final product was freeze dried and stored in desiccator.

l-PDLLA-COOH [1.9 g, ~ 0.01 x 10^{-3} mol, calculated with respect to its M_n (NMR) = 206 114 g mol^{-1}] and NHS (3.3 mg, 0.03 x 10^{-3} mol) was dissolved in DCM (25 mL) in the presence of EDC.HCl (10.9 mg, 0.057 x 10^{-3} mol) in a round bottom flask. The other reaction conditions were same as for ss-pLG.

2.2.4. Synthesis of ss-pLG coated gold nanoparticles

2% of Gel and ss-pLG solutions were prepared in H_2O prior to the synthesis of gold nanoparticles (AuNPs). All glassware were cleaned using freshly prepared aqua regia solution (HCl:HNO3 3:1) and then rinsed thoroughly with H_2O prior to use. 10 mL aqueous solution of $HAuCl_4$ (300 µL from 1 g in 100 mL stock, 0.1 mM) was taken in a beaker and 500 µL of $NaBH_4$ (0.1M) was added drop wise to the solution. The reaction mixture was stirred at 300 rpm for 10 min at room temperature. Then 500 µL of polymer solution was added to the mixture and it was further stirred for 30 min at room temperature. The red colored colloidal solution was further used for characterization.

2.2.5. Synthesis of hybrid polymer

10% of ss-pLG and gelatin was prepared in DMSO and water containing 10 mM hydrochloric acid (HCl) (300 µL in 3 mL). Then, GPTMS was added in the ratio of C-factors (the mole ratio of GPTMS/gelatin; molecular weight of gelatin was considered as 90 000 g/mol) 500 and stirred for overnight at temperature 60 °C.

2.3. Characterization of polymer

2.3.1. ^{1}H NMR study

^{1}H NMR spectra were recorded using Bruker Avance 500 MHz at room temperature in DMSO-d_6 as solvent, and were reported in parts per million (δ) from internal standard tetramethylsilane or residual solvent peak.

2.3.2. Gel Permeation Chromatography (GPC)

Younglin ACME 9000 GPC was used to determine the number-average molecular weight (M_n) and $\underline{M}_w/M_n$. Polymer solution in DMF was passed through two polystyrene gel columns [PL gel 5 mm 10E 4 Å columns (300 × 7.5 mm)] connected in series to a Younglin ACME 9000 Gradient Pump and a Younglin ACME 9000 RI detector with the flow rate 0.5 mL/min at 40 °C. The columns were calibrated using poly(methyl methacrylate) (PMMA) standard samples (Polymer Lab, PMMA Calibration Kit, MM-10)

2.3.3. X-Ray Diffraction (XRD) study

Powder XRD patterns were examined using an advance wide angle X-ray diffractometer with Cu-Kα radiation and a graphite monochromator (wavelength, λ =

0.154 nm, Rigaku, MiniFlex-600, Japan). Patterns were recorded at diffraction angle 2θ from 5 to 60° at a scanning rate of 10° min^{-1}

2.3.4. FTIR study

FTIR was done by Perkin Elmer IR spectrophotometer (Perkin Elmer Inc., USA) in the transmittance mode at room temperature from 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} using the KBr pellet technique. For ATR mode the polymer films having the dimension 10×10 mm and 1 mm thickness were used.

2.3.5. X-ray photoelectron spectroscopy (XPS)

XPS was used to characterize the surface properties of l-pLG and ss-pLG polmer film compared to the unmodified gelatin film. The spectra were measured using a XPS System (Kratos Analytical Instrument, Shimadzu group company, Amicus XPS UK.) with a monochromatic Mg-Kα (energy = 1486.71 eV) X-ray source. Samples were positioned at the electron take-off angle normal to the surface with respect to the analyzer. The spectra were recorded over a range of 0–1400 eV for both scaffold samples, followed by high resolution spectra for C1s, N1s and O1s. Peak areas were normalized within VISION software using atomic sensitivity factors for Mg- Kα anode and from these areas the carbon composition and elemental ratios were determined.

2.3.6. Thermogravimetric Analysis (TGA)

Thermo gravimetric analysis (TGA) was done using Mettler TGA thermo gravimetric analyzer from 40 to 800 °C with a heating rate of 20 °C/min under N_2 atmosphere

2.3.7. Differential Scanning Calorimetry (DSC) analysis

DSC was carried out by using Mettler STAR SW 10.00 instrument under nitrogen atmosphere. Instrument calibration was done with indium before use. The samples were first heated to 150 °C at 10 °C min^{-1} heating rate and then quenched to −50 °C. A heating rate of 10 °C min^{-1} was used for second heating run. Results were reported from the second heating run. The midpoint of the heat capacity change was taken as glass transition temperature (T_g).

2.3.8. Water contact angle measurement

Water contact angle on polymer coated cover glass was measured with Kruss K100 tensiometer and the associated software [64]. For this untreated cover glass was coated with both the 2% of polymers we prepared and contact angle measurement was performed.

2.3.9. Thioflavin T assay

0.3 mg/mL BSA was added into 0.5, 1.0 and 2.0% concentration of Gel and ss-pLG and the mixture was kept at 70 °C in shaking incubator at 150 rpm for the protein aggregation study. On the other hand BSA without polymer was taken as positive control, which was reported to produce fibril at 70 °C after 12 h, [65] and freshly prepared BSA was used as negative control. At various time interval sample was taken out and the respective study was performed.

1.2 mM of Thioflavin T (ThT) solution was prepared in PBS (pH 7.4) and filtered through a 0.22 mm filter. 160 µL of polymer sample and 40 µL of ThT were added into black 96 well plate and fluorescence was measured using microplate multimode reader

(Biotek, synergy H1, excitation 440 nm; emission 480 nm). All the experiments were done in triplicate.

2.3.10. Circular dichroism (CD) spectroscopy

All CD measurements were carried out with a CD spectropolarimeter, J-810 (Jasco, Japan), at 25 °C. A rectangular quartz cell with a path length of 0.1 cm was used. CD spectra of plain BSA, BSA in the presence of 2% Gel and ss-pLG, and 2% polymer without BSA were recorded, before and after 24 h incubation at 70 °C. The concentration of BSA was 0.3 mg/mL. Each CD spectrum represents an average of three scans. The baseline of the sample was corrected by subtracting the CD spectrum of solvent. The spectra were recorded in the range of 190–260 nm with a scan speed of 100 nm/ min. The data pitch and response time was 1.0 nm and 1 s, respectively.

2.3.11. AFM measurement

AFM was used to assess the morphology of BSA, before and after the aggregation reaction. 50 µL of BSA with polymer and without polymer were taken out after 12 h of incubation at 70 °C and solvent casted on a cleaned mica sheet. AFM images were taken using NT-MDT (Moscow, Russia). Images were captured in the semi contact mode.

2.3.12. UV-Visible spectrophotometry

0.3 mg/mL BSA was added into 0.5, 1.0 and 2.0% concentration of Gel and ss-pLG and the mixture was kept at 37 °C in shaking incubator at 150 rpm for 4 h. Experiments were conducted at 0 h and 4 h. The absorption spectra were recorded using Shimadzu (UV-1700) UV-Visible spectrophotometer. 3 mL of respective sample were taken in a quartz cuvette and scanned from 230 to 600 nm.

2.3.13. Spectrofluorometry

Multimode microplate reader (Biotek, synergy H1, USA) was used to record the fluorescence of tryptophan quenching. 100 µL of BSA with polymer and without polymer was added to black 96 well plate and emission spectra was documented at excitation of 290 nm.

2.3.14. Transmission Electron microscopy

5 µL of colloidal gold solution was coated onto copper coated carbon grid and dried in air at room temperature. Then grids were imaged under Philips CM-10 operated at an accelerating voltage of 100 kV

2.4. Protein adsorption study

Protein adsorption study was conducted with the thin film of both the polymers having thickness of 1 mm. The thin films (5×5 mm) of both the polymers were incubated in BSA protein solution, prepared in PBS after they were wet in PBS completely. To wet the polymer films, they were kept in 100% ethanol for 30 min followed by keeping them in phosphate buffered saline (PBS) for 1 h. The wetted films were then incubated in 1% BSA for 4 h in rotating shaker at 150 rpm. After incubation, films were thoroughly washed with PBS (3 times) to remove the less adsorbed and unbound BSA from the film. Proteins adsorbed onto the films were recovered by incubating the film in 1% SDS for 1 h followed by homogenization. Total amount of proteins adsorbed onto the film were estimated by micro BCA assay[66] (Pierce, USA)

2.5. Fabrication of 3D scaffolds

Scaffolds were fabricated by phase separation followed by solvent extraction technique[67]. Briefly, 12 % polymer solutions of gelatin grafted and gelatine ungrafted polymers were prepared in DMSO. Although, THF is very good solvent for fabricating scaffolds from PLLA/ PDLLA[68], gelatin grafted l-pLG and ss-pLG were not soluble in THF because of the hydrophilic nature. So, to avoid the structural changes in scaffolds by solvent system, we chose DMSO to dissolve both gelatin grafted and unmodified polymers. The prepared polymer solutions of 50 µL were then poured on to the wells of a 96 well plate and kept at -20 °C for overnight. There after the molds with the polymer solution was transferred to freeze dryer (LyoQuest 85, Telstar, Spain) and dried at -80°C for 4 days. The scaffolds were taken out from the wells and kept in vacuum desiccator until further use.

In case of hybrid scaffolds, 10% hybrid gel was prepared and the cured gel was further freeze dried.

2.5.1. Characterization of Scaffolds

2.5.1.1. Scanning Electron Microscopy (SEM)

Surface morphology of scaffolds was evaluated by Scanning Electron Microscopy (Zeiss Evo18, Germany). Small sections (5×5) of polymeric scaffolds were taken and gold sputtering was done using sputtering unit (Q150RES, Quorum). To analyse the scaffolds morphology of freshly prepared 3D Scaffolds from synthesized polymers were taken and coated by gold and observed. The pore sizes were calculated from SEM images using the Image J program.

The degradation property of scaffolds at different time point such as Day 3 and Day 7 was also studied using SEM. In brief, scaffolds were taken out and washed with PBS for 3 times. They were then dried by freeze dryer. The dried scaffolds were further taken and analysed for the changes in surface morphology.

The morphology of RBCs were also evaluated using SEM. The BSA amyloid fibril and BSA with polymer incubated RBCs were taken out from the shaking incubator after 4 h. They were washed with PBS 3 times. Then, RBCs were fixed with 1% glutaraldehyde for 15 min and washing was done for 3 times. The fixed RBCs were stored in 4 °C until the further characterization. Surface morphology of RBCs was evaluated by Scanning Electron Microscopy (Zeiss Evo18, Germany). The blood smear was prepared and gold sputtering was done using sputtering unit (Q150RES, Quorum).

2.5.1.2. *In vitro* degradation of 3D scaffolds

In vitro degradation experiment of the modified and unmodified scaffolds was conducted in, lysozyme containing PBS and PBS, since PBS acts as buffer and regulates the pH of solution and lysozyme will act as the physiological enzyme model. On the other hand, sodium azide containing tris HCl buffer at pH 8.6 also used for studying degradation in proteinase k containing medium. The concentration of Proteinase K (0.2 mg/mL) and lysozyme (0.3 mg/mL) were used. [69]

The absolute dry weight of the 3D scaffolds were measured before it is placed in the degradation medium. 5-6 mg of scaffolds were taken (Microbalance BM-22, AND, Japan) and incubated in lysozyme containing PBS and PBS. Then, the samples were placed in a orbital shaker with the constant rotation of 150 rpm at 37 °C. At a predetermined time interval, the scaffolds were taken from the medium, washed with

distilled water and freeze-dried. The percentage of degradation was calculated from the weight loss of the scaffolds by the formula[70],

$$D = (W_o - W_t)/W_t \times 100$$

where D is percentage of degradation, W_o denotes original weight of the scaffolds, while W_t is the weight at time t: Biodegradation experiment was repeated three times and the average value was taken as the percentage biodegradation.

2.5.1.3. Compression modulus

Compression modulus was performed using Instron 3369 at room temperature with the compression rate of 5 mm/min. Cylindrical scaffold samples prepared by freeze drying were used to measure the mechanical property.

2.5.1.4. *In vitro* biomolecule release study

Docetaxel (DTX) was kindly gifted by TherDose Pharma pvt. Ltd, India. Drug loaded scaffolds were prepared by mixing 12 % of polymers (l-PDLLA, ss-PDLLA, l-PG and ss-PG) with 3%(w/w) of DTX in DMSO, then they were freeze dried. The freeze dried scaffolds (6 mg) were put into 10 ml of PBS containing 0.5% tween 80 and kept in rotating shaking incubator at 37 °C. At various time intervals, 3 mL of PBS was withdrawn and replaced with fresh buffer. The concentration of DTX present in the samples was determined by measuring the absorbance at 230 nm in a UV–Visible spectrophotometer [71] (UV 1700, Shimadzu, Japan).

2.5.1.5. Silica release profile

The silica release profile from the hybrid scaffolds was analysed in cell culture media. The scaffolds having the dimension of 8 mm dia were put into 200 µL DMEM/FBS containing cell culture media. After day 1,3,5 and 7 the scaffolds were taken out and washed completely with PBS. Further, the scaffolds were analysed by FTIR and Energy-dispersive X-ray spectroscopy along with elemental mapping. All the experiments were done in triplicates.

2.6. Cell Culture

Preadipocytes 3T3-L1and fibroblasts L929 were kindly gifted by Dr. Sheetal Gandotra and Dr.Vivek Rao of CSIR-IGIB, New Delhi. Musculoskeletal cells C2C12, fibroblasts 3T3, preosteoblasts (MG-63) and hepatocytes Hep-G2 were procured from NCCS, pune, India. MC65 cells were kindly gifted by Prof. George M. Martin, University of Washington, Seattle. Cells were cultured in Dulbecco's modified eagle's medium (DMEM) (high glucose), with 10% fetal bovine serum (FBS) and 1% Penicillin streptomycin in a CO_2 incubator at 37 $^{\circ}$C with 5 % CO_2, 95 % humidity. During the culture, medium was changed every two days. MC65 cells were maintained in TC containing MEM media.

For the cell culturing on 3D scaffolds, scaffolds were initially kept in 100 % ethanol for 30 min and then sterilized under UV for 30 min. Then the scaffolds were soaked in cell culture media and kept in CO_2 incubator at 37 ^{o}C for 30 min. Cells were counted and concentrated into volume of 20-25 µL, further they were seeded into the scaffolds and kept in CO_2 incubator for 3 h to allow the cells to attach and settle into the scaffolds. After 3h of incubation, fresh media was added.

2.6.1. Cell adhesion and spreading analysis

To evaluate the cell adhesion and spreading on 2D polymer coated cover glass and 3D scaffolds, we used a model focal adhesion protein vinculin (Anti-Vinculin Alexa Fluor@488, ebioscience, USA) in combination with actin filament stain (Rhodamine Phalloidin, Thermo Fisher scientific, USA). Cell adhesion was assessed only in 2D, where 5×10^4 cells/ cover glass was seeded on to polymer coated cover glass. After 24 h, cells were fixed with 4% paraformaldehyde and immune staining was performed. To assess the cell spreading on the polymer coated cover glass, we seeded 5×10^4 cells/ cover glass. Cells were fixed and stained after 48 h. Cell spreading on 3D scaffolds was observed by seeding 50×10^3 cells / scaffold and the cells were treated with fluorescent stains after 48 h to check the interconnectivity of cells on the scaffolds.

2.6.2. Fluorescent staining of cell nuclei and actin filaments

The cell nuclei, vinculin, and F-actin were co-stained by DAPI, alexafluor@488 labelled anti-vinculin and rhodamine labelled phallodin respectively, to evaluate the morphology of cells on the films. For cell adhesion study, after 24 h of cell culturing on the cover glass, samples were fixed using 4 % formaldehyde for 30 min at 37 $^{\circ}$C and the cells were permeabilized with 0.1 % Triton X containing PBS for 5 min at 37 $^{\circ}$C. For staining, cells were first blocked with 1% BSA for 30 min at 37 °C, incubated with alexafluor@488 labelled anti-vinculin and Rhodamine-phalloidin for 1 h at 37 °C, and subsequently stained with DAPI for 30 min at 37 $^{\circ}$C. After the samples were mounted on glass slides, immunofluorescent images were obtained using a fluorescent microscope (Leica, Germany). Incase of cell spreading experiment, samples were

stained after 48 h of cell culturing and only rhodamine-phalloidine was used. Remaining protocols were same as for the cell adhesion experiment.

2.6.3. Cell viability and Proliferation

The fabricated scaffolds were studied for cells vability using MTT assay, where purple coloured formazan is usually formed after reduction of MTT (3-(4, 5 dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide) by mitochondrial dehydrogenase enzyme in live cells that is further solubilized in appropriate solvent and intensity of this solubilized formazan read using a spectrophotometer that indicates the number of live cells. UV sterilized scaffolds (5×5) in 96 well plates were seeded with 50×10^3 cells / scaffold (L929, 3T3, 3T3-L1, C2C12, MG63 and Hep-G2 cells) and incubated at 37 $^{\circ}$C, 5% CO_2 under humidified conditions for 1, 3, 5 and 7 days. After incubation of respective time period, scaffolds were thoroughly washed with PBS and transferred to the unused wells of 96 well plate. 100 µL of MTT solution (5 mg/ mL) in DMEM was added into the wells and incubated for 4 h. After 4 h, MTT solution was removed and 100 µL of iso-propanol was added and read at 570 nm in microplate reader (Synergy H1, Biotek, USA). Experiments were done in triplicates[72]. Cell numbers were normalized to 1 according to the observation of cell growth in 2D culture plate on 3rd day.

Cell viability was also evaluated by live/dead cell assay kit (Biotium, USA) where that detects intracellular esterase activity in live cells and compromised plasma membrane integrity in dead cells using a cell permeable calcein AM (Ex/Em: 494/517 nm) and the impermeable ethdium homodimer-III red dye (Ex/Em: 530/620 nm). 10×10^3 cells were seeded on to the scaffolds and grown for 7 days. Media were changed every two days. After 7 days, scaffolds were taken out from the culture media and cells were incubated

with live/dead cell assay kit. Scaffold samples were imaged under confocal laser scanning microscope (CLSM) (Leica LAS X 2.0.1.14392).

2.6.4. Neuroprotection Assay in MC65 Cells

MC65 cells were washed twice with PBS, resuspended in Opti-MEM, and seeded in 96-well plates (5×10^4 cells/well). Then 20 µL 0.5,1.0 and 2.0% of Gel and ss-pLG were then added into 200 µL media containing cells and cells were incubated at 37 °C under TC+ or TC- conditions for 48 h. Thus the concentration becomes 0.05, 0.1 and 0.2% of Gel and ss-pLG. After 48 h, media was removed and 10 µL of MTT (5 mg/mL in PBS) with fresh media was added and the cells were incubated for another 2 h. Cell medium was then removed, and the remaining formazan crystals produced were dissolved in DMSO. Absorbance was immediately recorded at 570 nm using a multimode microplate reader (Synergy H1, Biotek, USA).

2.7. Hemocompatability of scaffolds

2.7.1. Blood sample

Blood samples were obtained in a 15 mL centrifuge tube containing EDTA as an anticoagulant, from Blood Bank, Banraras Hindu University (BHU), Varanasi, India.

2.7.2. Hemolysis

The fabricated Scaffolds were cut into small pieces and soaked in PBS for 30 min. Then 0.2 mL of PBS diluted anitocoagulated blood (1:10) was added into the tubes containing scaffolds. They were then kept in orbital shaker incubator with constant rotation of 150 rpm at 37 °C for 1 h and 8 h.

For amyloid-induced hemolysis experiment, 300 µL of red blood cells (RBCs) were taken in separate microcentrifuge tubes and 30 µL of different concentration of BSA and BSA with polymer was added into the erythrocytes. They were then kept in orbital shaker incubator with constant rotation of 150 rpm at 37 ˚C for 4 h.

After incubation, scaffolds were taken out from the blood and centrifuged at 3500 rpm for 10 min. Hemoglobin released into the supernatant was read at 540 nm (Synergy H1, Biotek, USA). 1% Triton X-100 and PBS (pH 7.4) were used as positive and negative controls respectively. The percentage of hemolysis was calculated as follows[73].

$$\%\ of\ Hemolysis = \frac{A_{test} - A_{neg}}{A_{Pos} - A_{neg}} \times 100$$

where, A_{test}, A_{neg}, and A_{pos} are the absorbance values of the scaffold treated sample, negative control (PBS), and positive control (Triton X-100), respectively. All the experiments were done in triplicate.

2.7.3. Evaluation of erythrocyte membrane Integrity

Lactate dehydrogenase (LDH) enzyme released from the erythrocytes were photometrically detected after treating with scaffold samples by LDH commercial assay kit (Tulip Diagnostics, India) [74]. Anticoagulated blood was centrifuged at 1565 × g for 10 min to collect the erythrocytes. Erythrocytes were further washed with PBS for 3 times. Erythrocytes pellet from 15 mL of blood was diluted into 20 times and 0.2 mL of erythrocyte suspension was added into scaffold containing tubes. LDH release was observed after 1 h and 8 h treatment. LDH quantification reagent was added to the erythrocyte sample after the time point and the absorbance change (ΔA) till 3 min. was

read at 340 nm in microplate multimode spectrophotometer (Synergy H1, Biotek, USA).The concentration of LDH released was calculated using the following formula:

LDH activity in U/L (at 30^{o}C) = ΔA/min. ×3333

2.7.4. Activated Partial Throboplastin Time (APTT) and Prothrombin Time (PT) measurement

Platelet Poor Plasma (PPP) was prepared by centrifuging blood for 15 min at 1565 × g. 0.2 mL of PPP was added to each scaffold sample and incubated at 37 $^{\circ}$C for 10 min. For the APTT measurement, PPP was mixed with 100 µL of Liquiceilin reagent (Tulip Diagnostics, India) and incubated at 37 $^{\circ}$C for 3 min. After 3min, 100 µL of aqueous $CaCl_2$ (0.03 M) was added to the mixture, and the stop watch was started to record the clotting time. PT measurements were carried out by adding 200 µL of liqiplastin reagent (Tulip Diagnostics, India) to the PPP and then clotting time was noted down [75]. Each experiment was carried out in triplicate.

2.8. Subcutaneous *in vivo* implantation of scaffolds and their immune response

The animals were acclimatized for two weeks under conditions of controlled temperature (25±2 °C), constant humidity and 12-h light/dark cycle with free access to standard laboratory food and water. All *in vivo* experiments were conducted per the animal care protocols approved by Central Animal Ethical Committee at Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, India (Registration No. 542/02/ab/CPCSEA) in accordance with “Principles of Laboratory Animal Care.” We used female Charles–Foster (CF) rats having the weight of 220-220 g body weight. The rats were subcutaneously implanted with acellular Gel, ss-PDLLA, ss-pLG and the

hybrid h-ss-pLG, h-Gel scaffolds that have the specific dimensions (8 mm in diameter, 2 mm in height). The study was conducted for two time points; week 1 and week 4. The weight loss was observed for the course of the experiment, and no animal death was documented within the experimental period. After 1 and 4 week of implantation, scaffolds were retrieved from the implanted site with the overlying native tissues. The scaffolds with tissues were put into 10% formaldehyde for 48 h then dehydrated through a series of graded ethanol, cleared with xylene, and embedded in paraffin wax. For histological studies, tissue sections were prepared. The slices were then deparaffinized, cleared, and stained further with hematoxylin and eosin (H&E, Sigma-Aldrich, USA) following the manufacturer's instructions, and sections were assessed for the infiltration of inflammatory cells.

2.9. Statistical Analysis

All measurements were reported as mean ± standard deviation (SD), n=3 with a confidence level of 95%. Differences were statistically tested against a null hypothesis of no difference between samples using one way ANOVA and two way in GRAPH PAD PRISM 5.