CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials Used for Fabrication of the Fibrous Scaffolds

- Polycaprolactone (PCL) : Aldrich, USA
- Dichloromethane (DCM) : Aldrich, USA
- N,N'-dimethylformamide (DMF) : Lab-Scan (Asia)

3.1.2 Materials Used for Surface Modification

- 1,6-hexamethylenediamine (HMD) : Aldrich, USA
- N,N'-disuccinimidyl carbonate (DSC) : Aldrich, USA
- Ninhydrin : Aldrich, USA
- Bovine Serum Albumin (BSA) : Sigma, USA
- Triethylamine (TEA) : Sigma, USA
- Ethanol : Lab-Scan (Asia)
- Isopropanol (IPA) : Lab-Scan (Asia)
- 1,4-dioxane : Lab-Scan (Asia)
- Dimethysulfoxide (DMSO) : Lab-Scan (Asia)
- Phosphate Buffer Saline (PBS)

3.1.3 Materials Used for Cell Culture

3.1.3.1 Model Cells

Model cells in this study were mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1).

3.1.3.2 MC3T3-E1 cells culture medium

MC3T3-E1 cells were cultured in Minimum Essential Medium with Earle's Balanced Salts (MEM; Hyclone, USA), supplemented by 10 % Fetal Bovine Serum (FBS; Biochrom Ag, Germany), 1 % L-glutamine (Invitrogen Corp., USA), and 1 % antibiotic and antimycotic formulation [containing penicillin G Sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA)]

3.2 Equipment

3.2.1 Equipment for Electrospinning Process

- High voltage power supply from Gamma High Voltage Research Inc. (Ormond Beach, Florida), model D-ES30ON/M692 DC was used to generate positive DC voltage.
- Syringe with volume size of 20 ml Stainless steel needle with gauge number 20 (or the inner diameter of 0.91 mm).
- The rotating drum which covered with aluminum sheet which has the width and outer diameter about 14 and 15 cm, respectively.

3.2.2 Equipment for Characterization of Materials

- UV-Vis spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan)
- Microplate reader model Infinite 200 PRO NanoQuant Multimode Microplate Reader from Tecan AG
- Fourier-transformed infrared spectrometer (FR-IR); attenuated total reflection (ATR-FTIR; Thermo Nicolet Nexus 670; resolution of 4 cm^{-1} and 128 scans)
- Contact angle goniometre (KRUSS Gmbh Germany; Model: DSA10-Mk2 T1C)
- Scanning electron microscope (SEM; A JEOL JSM 5410LV0)

3.2.3 Equipment for Cell Culture Study

- A Thermospectronic Genesis10 UV-visible spectrophotometer
- Universal Testing Machine (UTM; SHIMADZU, EZ-SX)
- pH Meter (DENVER Instrument; Model 220)

3.3 Methodology

3.3.1 Preparation of Electrospun Fiber Mats

1. The spinning solution was prepared by dissolving the 12% (w/v) of PCL 50:50(v/v) DCM/DMF.

2. The as-prepared solution was continuously stirred until clear solution was obtained.

3. The solution was fed into the glass syringes fitted with needles.

4. The Al sheet was wrapped around the rotating cylinder (rotating speed : 50 rpm) as a collector.

5. The distance between needle's tip and collector was fixed at 15 cm.

6. A voltage dc power supply was used to generate a high dc potential for PCL at 21 kV.

The morphological appearance and size of the individual fibers of the scaffolds were determined by a JEOL JSM-5200 scanning electron microscope (SEM). Fibrous scaffolds were dried in air overnight to remove solvent. Each specimen was coated with a thin layer of gold by using a JEOL JFC-1100E ion sputtering device prior to SEM observation. The average diameters of as- spun fibers were determined by measuring the diameters at least 100 points from 5 SEM images are statistically analyzed using SemAphore 4.0 software. The arithmetic mean values of the individual fiber diameters in the PCL fibrous scaffolds were determined to be 0.50 ± 0.05 μm.

3.3.2 Surface Modification of Fibrous Scaffolds via Aminolysis and Immobilization of Protein

The PCL fibrous scaffolds were immersed in ethanol/water (1:1, v/v) for 2-3 h to clean the fiber surface and then washed with large amount of distilled water. The PCL scaffolds were subsequently immersed in 0.2 g/ml HMD/IPA solution of varying reaction time (10, 30, 60, 120, 180 min) at 30°C. The aminolyzed fibrous scaffolds were then rinse with distilled water for 24 h at room temperature to remove unreacted HMD, then dried in vacuo at room temperature.

Aminolyzed PCL fibrous scaffolds were then activated by immersing obtained fibrous scaffolds in 0.1M DSC/DMSO in the presence of 0.1M TEA for an hour at ambient temperature, respectively. Followed by rinsing with large amount of distilled water.

The scaffolds were then directly transferred to 3 mg/ml of BSA/PBS at ambient temperature for 24 h Immobilized fibrous scaffolds were then rinsed by soaking in distilled water for 24 h The samples were dried in vacuo at room temperature.

By reaction with 1,6-hexamethylenediamine (HMD), amino groups can be covalently introduced onto the surface of the PCL fibrous scaffolds. One amino group (NH_2) of HMD reacts with an ester group (-COO-) of polymer to form the amide linkage (-CONH-), while another amino group is unreacted and free for the further reaction. These free NH_2 groups can be used as active sites through which proteins like bovine serum albumin (BSA) can be bonded to the surface using N,N'-disuccinimidyl carbonate (DSC) as a coupling agent. However, to avoid aggregration, two-step procedure was employed. The attached amino groups had been first activated with DSC which N-hydroxysuccinimide being lost from the reaction and the as-formed succinimidyl esters were later reacted with respective biomolecule, BSA, with N-hydroxysuccinimide again being cleaved from the reaction. The chemical pathway for the immobilization of biomolecule on the surface of polymeric fibrous scaffolds is summarized in the Figure 3.1.

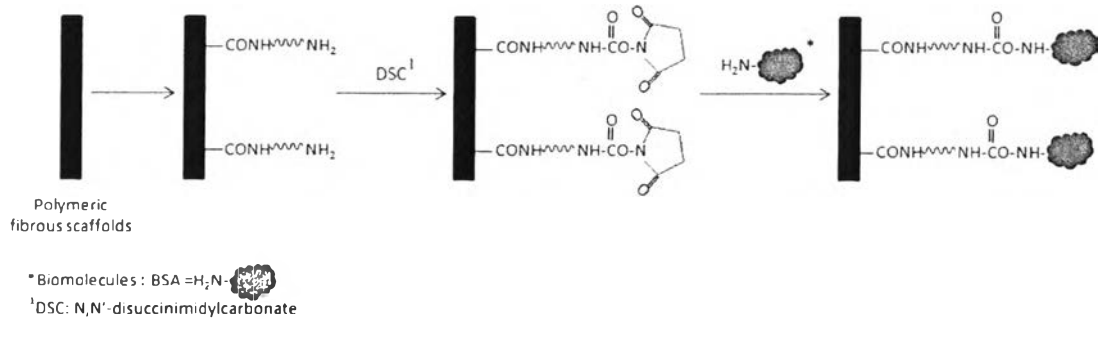

Figure 3.1 The chemical pathway for the immobilization of protein onto polymeric fibrous scaffolds.

3.3.3 Degradation Experiment

The fibrous scaffolds which product from PCL via electrospinning technique were examined the degradation in the absence and presence of enzyme lipase from *Pseudomonas sp.* In phosphate buffer saline (PBS) solution. Briefly, the polymeric scaffolds from each condition had been separately immersed in 5 ml of 0.1M PBS, pH 7.4 with or without lipase (*Pseudomonas sp.*, 45 units/l) for 4 weeks. The buffer solution containing lipase was replaced every 84 h. so that that enzyme activities were maintained at a constant level throughout the experiment. The samples were taken out at the different time intervals, and washed thoroughly with distilled water and dried in room temperature for 24 h. and then were dried in vacuum for 48 h. The weight remaining, morphological properties, and thermal properties of the scaffolds were monitored to determine the degradation of the fibrous scaffolds.

3.3.4 Fibrous Scaffolds Characterization

3.3.4.1 Contact Angle Measurement

A contact angle goniometer (KRUSS Gmbh Germany; Model: DSA10-Mk2 T1C) equipped with a Gilmont syringe and a 24-gauge flat-tipped needle was used to determine contact angles of a water drop on the surfaces of both the neat and the surface-modified polymeric scaffolds. The measurements were carried out by the sessile drop method in air at room temperature in pentplicate on different areas of each sample.

3.3.4.2 Protein Absorption Test

The protein on the surface of protein-adsorbed films was detached from the polymer surface by immersing the sample in 2 mL of 1% (w/v) sodium dodecyl sulfate (SDS) aqueous solution for 30 min. The concentration of protein dissolved in SDS solution can be determined. The protein adsorption test was carried out based on bicinchoninic acid method by using Pierce BCA protein assay kit. The SDS solution with dissolved protein of 25 μL was pipette into 96-well plate. The BCA working solution of 200 μL was then 16ipette into each well. The mixture of protein in SDS solution and BCA working reagent in the plate were shook thoroughly on a plate shaker for 30 seconds in order to mix the solution well.

The plate was covered and incubated at 60°C for 30 min. Then, the plate was cooled to room temperature. Microplate reader model Infinite 200 PRO NanoQuant Multimode Microplate Reader from Tecan AG was used to determine the optical density of samples at the wavelength of 562 nm in BCA assay.

3.3.4.3 Weight Loss of Fibrous Scaffolds

The weight loss of fibrous scaffold was measured and calculated by the following equations

$$\text{Weight loss (\%)} = \frac{W_f - W_i}{W_i} \times 100$$

Where W_i is the initial weight of the scaffolds and W_f is the weight of the scaffold at the given degradation time point, immersed in 0.1 M PBS solution. The weight loss was calculated from those of five samples in each group.

3.3.4.4 Scanning electron microscopy (SEM)

Electrospun fibrous structures were sputter coated with gold by using a JEOL JFC-1100E device prior to SEM observation. Their morphologies were observed by SEM (JEOL JSM-5200, Japan) at an accelerating voltage of 2 kV.

3.3.4.5 Thermal Propetries of Fibrous Scaffolds

NETZSCH DSC 204 F1 differential scanning calorimeter (DSC) was used to investigate the crystallization behavior of the fibrous scaffolds before and after degradations. Each sample of about 3 mg was first heated from room temperature to above melting temperature of those scaffolds about 50°C (the melting temperature of PCL are about 60°C) at a rate of 10°C min^{-1}(HEAT1). After that the sample was cooled down to 20°C at a rate of -10°C min^{-1}(COOL) and then reheated to the same temperature at the same rate (HEAT2). The apparent degree of crystallinity of the each fibrous scaffold was assessed from the enthalpy of fusion as obtain from HEAT1.

Crystallization is an exothermal transition. It occurs at temperatures slightly lower than the melting point of the polymer. The crystallization of a polymer depends on the capacity of its chains to move and form a crystalline

structure. The H_c is indicative of polymer chain length. The crystallization temperature and enthalpy are defined as the maximum of the crystallization peak and the area under the crystallization curve respectively.

Melting is and endothermal transition, the melting point is defined as the melting point is defined as the minimum of the melting peak. The melting point of the PCL used for this study ranges between 60°C. The heat of fusion is the area under the melting peak. Only the crystalline regions of the PCL have a melting temperature.

The heat of fusion is thus used to compute the percentage of crystalliniy, X_c calculated as:

$$X_c(\%) = \frac{\Delta H_m}{\Delta H_{m^\circ}} x100$$

Where, ΔH_m was the measured enthalpy of melting and ΔH_{m° was the enthalpy of melting of 100% crystalline polymer. For PCL, ΔH_{m° = 139.5 J/g (Pitt *et al.*, 1981)

3.3.4.6 Mechanical Characterization

The fibrous structures were cut into 1 cm × 8 cm rectangular shapes and tested for tensile property with an uniaxial tensile tester. Sample width and thickness were measured using a micrometer before being gripped within the tensile tester clamps of the Instron. Samples were tested to failure with a 500 N load cell and 10 mm min^{-1} crosshead speed. The modulus was determined by comparing two stress-strain points in the initial linear region of the curve. The ultimate tensile strength (UTS) was taken as the highest stress point reached on the curve. Five replicates of each sample type were tested per time point.

3.3.4.7 pH of degradation buffer

The pH of the degradation buffer in every test tube was measured with a pH meter twice a week.

3.3.5 Biological Characterizations

Osteoblast (MC3T3-E1) cell lines are used. MC3T3-E1 cells were cultured in α-MEM medium supplemented with 10%FBS, 1%L-glutamine and 1% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, and amphotericin B). The medium was replaced every 2 days and the cultured cells were maintained at 37°C in a hymidified atmosphere containing 5% CO_2.

3.3.5.1 Cell Culturing

The 15 mm-diameter circular specimen was put into 24-well tissue-culture polystyrene plate (TCPS). The plate was sterilized with 70% ethanol for 30 min. The specimens were washed with autoclaved deionized water, PBS and immersed in SFM overnight. The specimens were pressed with a metal ring (about 12 mm in diameter) for the complete contact between the plate and the specimens. The MC3T3-E1 cells were trypsinized [0.25% trypsin containing 1m MEDTA (Invitrogen Corp.,USA)] and counted by a hemacytometer (Hausser Scientific, USA). The density of seeded cells on the specimen was 40,000 cells/cm^2. The well of TCPS without any specimen was used as a control. The cultured cells were kept at 37°C under 5% CO_2.

3.3.5.2 Determination of Cytotoxicity

Each of the neat, aminolyzed, activated and BSA-immobilized fiber mat specimens were determined for their indirect cytotoxicity and TCPS was used as a control. The extraction medium was prepared by immersing the sample in 24-well plate containing a serum-free medium (SFM) which was kept at 37°C under 5% CO_2 for 1, 3 and 7 d. 40,000 MC3T3-E1 cells/well were separately culture in another 24-well plate for 1 d to allow cells to attach to the plate. The cells were then starved with SFM for another 1 d. After the starvation, the culture medium was replaced with the prepared extraction medium. The cells were cultured in the extraction medium for 24 h before the MTT assay was performed to determine the amount of viable cells.

3.3.5.3 MTT assay

MTT assay is a quantitative analysis to determine cell viability. Living cells are able to use the enzyme called succinate dehydrogenase in mitochondria to react with the yellow MTT reagent, producing purple formazan product whose amount is proportional to the number of viable cell. Dimethylsulfoxide (DMSO) was used to dissolve the purple formazan product inside cell to find the absorbance at 570 nm.

In order to perform MTT assay, the culture medium was removed out from each well and incubated with MTT solution at 37°C for 30 min then the MTT solution was removed afterwards. A buffer solution, containing 900 μL/well of dimethylsulfoxide (DMSO) and 125 μL/well of glycine buffer (pH 10), was added in each well to dissolve purple formazan crystal. The solution was shaken for 10 min then filled in the cuvette and placed into a UV-Vis spectrophotometer (Thermospectronic Genesis 10 UV-Visible spectrophotometer) to measure the absorbance at 570 nm. The absorbance was related to the number of living cells.

3.4 Statistical Analysis

All the results are expressed as a mean ± standard deviation (SD). ANOVA single factor analyses were done and the values of $p < 0.05$ were considered significant. Each parameter was repeated on 3 samples (n=3).