CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials Used for Film Casting

- Poly(ε-caprolactone) (PCL, M_n = 80,000 g/mol) : Aldrich
- Chloroform : Lab-Scan (Asia)
- Tetrahydrofuran (THF) : Burdick & Jackson
- Acetone : Lab-Scan (Asia)
- Ethanol : Merck
- Sodium hydroxide (NaOH)

3.1.2 Materials Used for Surface Modification

- 1,6-hexamethylenediamine (HMD) : Aldrich
- Isopropanol (IPA) : Burdick & Jackson
- *N,N'*-disuccinimidyl carbonate (DSC) : Aldrich
- Dimethylsulfoxide (DMSO) : Lab-Scan (Asia)
- Triethylamine (TEA) : Sigma
- Bovine serum albumin (BSA) : Sigma
- Phosphate buffer saline (PBS) : Gibco
- 1,4-dioxane : Fisher Sciencific
- Sodium dodecyl sulfate (SDS) : Sigma

3.1.3 Materials Used for Cell Culture

3.1.3.1 Model Cells

Mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1) were used as model cells.

3.1.3.2 MC3T3-E1cells 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))

3.1.3.3 Cell Culture Study

- Methylene Blue
- Ethanol : J. T. Beaker, USA
- Hydrochloric acid
- 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) : USB Corporation, USA
- DMEM without phenol red : GibThai Co., Ltd.
- Dimethylsulfoxide (DMSO)
- Trypsin-EDTA solution : GibThai Co., Ltd.
- L-glutamine : GibThai Co., Ltd.
- Fetal Bovine Serum : Sorali, Brazil
- Bicinchoninic acid protein assay (BCA) : Pierce, USA
- *p*-nitrophenylphosphate (PNPP) : Zymed Lab., USA
- L-ascorbic acid : Sigma, USA
- β-glycerophosphate : Sigma, USA
- Dexamethasone : Sigma, USA
- Cetylpyridinium chloride : Sigma, USA

3.2 Equipments

3.2.1 Differential Scanning Calorimeter (DSC)

Differential scanning calorimeter (Perkin-Elmer; Model: DSC7) was used to determine the melting point of the films.

3.2.2 Contact Angle Measurements

Contact angle goniometer (KRUSS Gmbh Germany; Model: DSA10-Mk2T1C) was used for the measurement of water contact angles on the surfaces.

3.2.3 Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR)

Attenuated total reflectance-Fourier transform infrared spectrometer (ATR-FTIR; Thermo Nicolet Nexus 670) was used to determine functional groups on the surfaces.

3.2.4 Scanning Electron Microscope (SEM)

Scanning electron microscope (SEM; A JEOL JSM 5410LV0) was used to investigate surface topography of the film.

3.2.5 Atomic Force Microscope (AFM)

Atomic Force Microscope (AFM; PARK; Model: XE-100) was used to examine the topography of PCL membrane.

3.2.6 UV-Visible Spectrophotometer

UV-Visible spectrophotometer (Thermospectronic Genesis 10) was used to determine the optical density of the samples in MTT assay.

3.2.7 X-Ray Diffractometer (XRD)

X-ray diffractometer was used to determine degree of crystallinity of the films.

3.2.8 Microplate Reader

Microplate reader model Infinite 200 PRO NanoQuant Multimode Microplate Reader from Tecan AG was used to determine the optical density of samples in BCA assay.

3.3 Methodology

3.3.1 The Effect of Degree of Crystallinity to Protein Adsorption

3.3.1.1 Preparation of Biodegradable Films

Polycaprolactone (PCL) film was prepared by dissolving 0.3 g of PCL pellet in 5 mL chloroform then casted uniformly on a clean glass Petri Dish with 10 cm diameter. The solvent was evaporated for 24 h at room temperature. The 6 % (w/v) translucent film was obtained.

3.3.1.2 Inducing Different Degree of Crystallinity on The Films

The PCL films were annealed at 40 °C. The annealing time was varied to be 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, and 24 h to create different degree of crystallinity.

3.3.2 The Effect of PCL Surface Topography to Protein Adsorption

3.3.2.1 Preparation of Polycaprolactone Films

Polycaprolactone (PCL) film was prepared by dissolving 0.3 g of PCL pellet in 5 mL solvent (i.e. chloroform, acetone, tetrahydrofuran (THF), 20:80 (v/v) ethanol:THF, 30:70 (v/v) ethanol:THF, and 40:60 (v/v) ethanol:THF) then casted uniformly on a clean glass Petri Dish with 10 cm diameter. The solvent was evaporated for 24 h at room temperature. The 6 % (w/v) translucent film was obtained.

3.3.2.2 Surface Modification of Polycaprolactone Film

The surface of PCL film can be modified by 2 methods: aminolysis and hydrolysis.

3.3.2.2.1 Surface Modification of PCL Film via Aminolysis

The PCL film was cut into a 1.5 cm-diameter circle then immersed in ethanol/water (1:1 v/v) solution for 2-3 h to remove oil and dirt on the film surface. The clean film was washed with a lot of deionized water. The aminolysis was done by further immersed the film in 1.5 M 1,6-hexamethylenediamine (HMD)/isopropanol (IPA) solution for 8 h at 37°C. The aminolyzed film was rinsed with deionized water for 24 h at room temperature and vacuum dried at 30°C until it reached the constant weight.

3.3.2.2.2 Surface Modification of PCL Film via Hydrolysis

The PCL film was cut into a 1.5 cm-diameter circle and soaked into 1M or 5 M NaOH solution for 3 h at room temperature. The film was washed with deionized water for 3 times then immersed in deionized water overnight to remove NaOH residue and vacuum dried at 30°C to the constant weight.

3.3.2.3 Activation of The Amino Group on Aminolyzed Film

The amino groups on aminolyzed PCL film were activated by immersing the aminolyzed film in 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/dimethylsulfoxide (DMSO) solution with 0.1 M triethylmine for 1 h at room temperature. The activated aminolyzed PCL film was then rinsed with a large amount of deionized water.

3.3.2.4 Immobilization of Bovine Serum Albumin

The activated aminolyzed film was subsequently immersed in 3 mg/mL of bovine serum albumin (BSA)/phosphate buffer saline (PBS) solution for 24 h at room temperature. The biomolecule-immobilized film was soaked in deionized water for 24 h and dried in vacuo at room temperature.

3.4 Characterization

3.4.1 Differential Scanning Calorimeter (DSC)

Differential scanning calorimeter can be used to determine heat of reactions by the controlled temperature profile. The temperature change of a sample was compared to that of the reference material with the same heating program. The change in physical state or phase of transition could be observed. The samples were run from 10 °C to 120 °C at the rate of 10 °C/min in N_2 atmosphere.

3.4.2 Contact Angle Measurement

Contact angle goniometer (KRUSS Gmbh Germany; Model DSA10-Mk2T1C) with a Gilmont syringe and a 24-guage flat-tipped needle was used to examine the water contact angle on the surface of the films which would provide the information on hydrophobicity of the film. The measurement was taken at room temperature.

3.4.3 Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR)

Attenuated total reflectance-Fourier transform infrared spectrometer (Thermo Nicolet; Model: Nexus 670) was used to determine the functional groups on the surface at the resolution of 4 cm^{-1} for 128 scans.

3.4.4 Scanning Electron Microscope (SEM)

The surface topography of the film and cell morphology were investigated by scanning electron microscope (SEM) model JEOL JSM 5410L V. Before using SEM, each sample was prepared by coating with a thin gold layer using JEOL-JFC-1100E ion sputtering device.

3.4.5 Atomic Force Microscope (AFM)

Atomic Force Microscope (AFM; PARK; Model: XE-100) was used to scan the surface of PCL membrane over a sampling area of 40 x 40 μm^2. The images were made in IC-AFM mode. The measurements were performed at 25°C at 30-40% relative humidity in air.

3.4.6 UV-Visible Spectrophotometer

UV-Visible spectrophotometer (Thermospectronic Genesis 10) was used to determine the optical density of the samples at the wavelength of 570 nm in MTT assay.

3.4.7 X-Ray Diffractometer (XRD)

X-ray diffractometer was used to determine degree of crystallinity of the films. In the x-ray spectrum, it showed a broad peak indicating the amorphous region, and the sharp peaks on the broad peak are related to the crystalline region. The area under crystalline peaks and amorphous peak can be used to determine degree of crystallinity.

$$\% \text{ Crystallinity} = \frac{\text{Area of crystalline phase}}{\text{Area of crystalline phase + Area of amorphous phase}} \times 100 \qquad (3.1)$$

3.4.8 Protein Adsorption 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 pipetted into 96-well plate. The BCA working solution of 200 µL was then pipetted 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.

3.5 Biological Characterization

Osteoblast cell lines (MC3T3-E1) was used to determine the biological response of PCL film. They were cultured in α-MEM medium which was changed every 2 days. The cultured cells were kept at 37°C under 5% CO_2.

3.5.1 Cell Culturing

The 15 mm-diameter circular film 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 for the complete contact between the plate and the specimens. The MC3T3-E1 cells were trypsinized and counted by a hemacytometer. 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.

3.5.2 Determination of Cytotoxicity

Each of the neat PCL, aminolyzed PCL, BSA-immobilized PCL, and BSA-adsorbed PCL casting from different solvent 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.

3.5.3 Cell Attachment and Proliferation

Films were immersed in SFM overnight and then SFM was removed out. After which, 40,000 MC3T3-E1cells and 0.5 mL of 10% MEM were pipetted into each well that contained the prepared film as well as pipetted into the empty TCPS to be used as a control. The plate was kept at 37°C under 5% CO_2. The cell adhesion was observed at 4, 16 and 24 h of the cell culture time and the cell

proliferation was observed at 1, 2 and 3 d of cell culture time. MTT assay was used to determine the number of viable cell and SEM was used to see the cell morphology.

3.5.4 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.

3.5.5 Cell Morphological Observation

The cell culture medium was removed and the cell-cultured samples were rinse with PBS twice before fixing the cells with 500 μL/well of 3% glutaraldehyde solution (from the dilution of 50% glutaraldehyde solution using PBS) for 30 min. The cell-cultured samples were rinsed again with PBS. After the cell fixation, a series of ethanol solution (i.e. 30, 50, 70, 90 and 100%, respectively) was used to dehydrate the samples for 2 min at each concentration. The samples were dried in air. When they were completely dried, the samples were mounted on an SEM stub and coated with thin layer of gold. The observation on a JEOL JSM 5200 scanning electron microscope (SEM) was done to observe the cell morphology.

3.5.6 Mineralization

Alizarin Red-S can be used to identify calcium in the tissue in which calcium is able to form a complex with Alizarin red-s in chelation process. MC3T3-

E1 of 40 000 cells were cultured on the sample for 21 days to observe the production of mineralization. Cells were cultured in 10 % MEM culture medium for 3 days. After which, they were cultured in the new culture medium. The new culture medium could be prepared from 10 % MEM in presence of 1 μL/ml of ascorbic acid, 10 μL/ml of β-glycerophosphate, and 1 μL/ml of dexamethazone. The culture medium was changed every 2 days. The 1 % Alizarin red-S solution was prepared from dissolving Alizarin red in distilled water and the pH was adjusted to 4.1-4.3 by 10 % ammonium hydroxide. After 21 days, the cells were washed twice with autoclaved PBS and fixed with 500 μL of cold methanol for 10 min. The cells were then stained with 370 μL/well of 1 % Alizarin red-S solution for 3 min. The Alizarin red-S was removed and the cells were washed with distilled water. After which, they were dried at room temperature. The images of the culture were captured. In order to extract the stain of Alizarin red-S, the 1000 μL of 10 % cetylpyridinium chloride in 10 mM sodium phosphate were added for 20 min. The optical density could be obtained at the wavelength of 570 nm by using UV-vis spectrophotometer (Thermo Spectronic Genesis 10).

3.6 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).