CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Polymers and Solvents Used for Film Casting

- Polycaprolactone (PCL, M_n = 80,000 g/mol) : Aldrich
- Chloroform : Lab-Scan (Asia)
- Tetrahydrofuran (THF) : Burdick & Jackson
- Ethanol : Merck

3.1.2 Reagents 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
- Sodium dodecyl sulfate (SDS) : Sigma

3.1.3 Materials Used for Cell Culture Experiment

3.1.3.1 Cell Line

Mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1) were used to investigate the biocompatibility of PCL films.

3.1.3.2 MC3T3-E1cells culture medium

- Minimum Essential Medium (MEM): Hyclone
- 10 % Fetal Bovine Serum (FBS) : Biochrom Ag
- 1 % L-glutamine : Invitrogen Corp.
- 1 % Antibiotic and Antimycotic : Invitrogen Corp.
  formulation (containing penicillin G Sodium, streptomycin sulfate, and amphopericin B

3.1.3.3 Cell Culture Study

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

3.2 Equipments

3.2.1 Contact Angle Measurements

Surface wettability of the materials was measured by the contact angle goniometer (KRUSS Gmbh Germany; Model: DSA10-Mk2T1C). The values were evaluated from seven droplets of distilled water with a Gilmont syringe, a 24-guage flat-tipped needle, 5 μm of volume, temperature 23 $\pm$ 2 °C and humidity 60 $\pm$ 5 %. The water contact angle on the surface of the film obtained a result of hydrophobicity on the surface.

3.2.2 Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer

The functional group on the surface of the materials were identified by attenuated total reflectance-fourier transform infrared spectrometer (ATR-FTIR;

model EQINOX 55) over a range between 4000-600 cm^{-1} at 4 cm^{-1} resolution, averaging 64 scans.

3.2.3 Scanning Electron Microscope (SEM)

Surface morphology of the PCL films was investigated by scanning electron microscope (SEM; A JEOL JSM 5410LV0). Before observing SEM, the samples were sputter coated with platinum. The coated samples were placed in the vacuum chamber of the SEM and visualized under the SEM at an accelerating voltage of 2 kV.

3.2.4 Atomic Force Microscope (AFM)

Surface topography of PCL films was observed using Atomic Force Microscope (AFM; Nanoscope® IIIa Scanning Probe Microscope Controller). The microscope was operated in a tapping mode (scan size 10 x 10 μm^2) with a V-shape cantilever. The average deviation (Ra) values were calculated, and 3D images of the surfaces were analyzed by AFM.

3.2.5 Microplate Reader

The optical density of the samples was determined by microplate reader (Infinite 200 PRO NanoQuant Multimode Microplate Reader) in MTT, BCA assay and ALP analysis. The UV absorbance values are proportional to the number of cells (MTT assay), to the protein concentration (BCA assay) and the amount of ALP that can be converted by calculating standard calibration curves.

3.3 Methodology

3.3.1 The Effect of PCL Surface Topography to Protein Adsorption

The volume of the solution to cast on the Petri Dish was 20 mL for preparation smooth surface and 8 mL for preparation rough surface that provided the nearly thickness about 188-230 μm.

3.3.1.1 Preparation Smooth Surface of PCL Films

Polycaprolactone film was dissolved in chloroform (7.2 g of PCL pellet in 120 mL the solvent) for 4 hrs to homogenous solution then casted uniformly in 20 mL of volume on a clean glass Petri Dish with 10 cm diameter. The

solvent was evaporated for 24 hrs at 25 ± 2 °C. The 6 % (w/v) translucent film was obtained.

3.3.1.2 Preparation Rough Surface of PCL Films

Polycaprolactone (PCL) film was dissolved in 40:60 (v/v) EtOH:THF (4.8 g of PCL pellet in 80 mL the solvent) for 12 hrs to homogenous solution then casted uniformly in 8 mL of volume on a clean glass Petri Dish with 10 cm diameter. The solvent was evaporated for 24 hrs at 25 ± 2 °C. The 6 % (w/v) porous film was obtained.

3.3.1.3 Surface Modification of Polycaprolactone Film

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

3.3.1.4 Activation of The Amino Group on Aminolyzed Film

The surface of aminolyzed PCL film was activated by immersing in 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/dimethylsulfoxide (DMSO) solution with 0.1 M triethylmine for 1 hrs at 25 ± 2 °C then rinsed with a lot of deionized water to obtain activated aminolyzed PCL film

3.3.1.5 Adsorption of Bovine Serum Albumin and Collagen

Adsorption of bovine serum albumin and collagen was done by immersing the activated aminolyzed film in 3 mg/mL for 0.5 mL of bovine serum albumin (BSA)/phosphate buffer saline (PBS) for 24 hrs at 25 ± 2 °C and 0.1 mg/mL of Collagen /phosphate buffer saline (PBS) solution for 24 hrs at 3-8 °C. The biomolecule-immobilized film was immersed in deionized water for 24 hrs at 3-8 °C and dried.

3.3.1.6 Pre-adsorption of Bovine Serum Albumin and Adsorption of Collagen

The activated Aminolyzed PCL film was immersed in 0.2, 1.5 and 3 mg/mL BSA/PBS for 24 hrs at 25 ± 2 °C before and dried. The immobilized BSA PCL film was soaked in 0.1 mg/mL collagen type I for 24 hrs at 3-8 °C after that the biomolecule-immobilized film was immersed in deionized water for 24 hrs at 3-8 °C and dried.

3.3.1.7 Protein Adsorption Test

Determination the amount of the protein-adsorbed films was done by immersing the specimens in 2 mL of 1% (w/v) sodium dodecyl sulfate (SDS) aqueous solution for 30 min. SDS solution was detached the protein-adsorbed on the specimens surface. The protein adsorption test was carried out based on bicinchoninic acid method by using Pierce BCA protein assay kit. The BCA working solution of 200 μL was pipetted into 96-well plate then the SDS solution with dissolved protein of 25 μL was pipetted into each well. The mixture of protein in SDS solution and BCA working reagent in the plate were shook on a plate shaker for 30 seconds to homogenous solution. The plate was covered and incubated at 60 °C for 30 min. After that, the plate was cooled to at 25 ± 2 °C and measured absorbance at 562 nm within 10 min. Microplate reader was used to determine the amount of protein-adsorbed on the sample surface.

3.3.2 Biological Experiments

Mouse Calvaria-Derived Preosteoblastic Cells (MC3T3-E1) was used as reference cell line. The MC3T3-E1 cells were cultured in α-MEM medium which was changed every 2 days and incubated at 37°C under 5% CO_2.

3.3.2.1 Cell Culturing

The film specimen was cut in a 15 mm-diameter circular and put into 24-well tissue-culture polystyrene plate (TCPS) then sterilized with UV radiation for 20 min. The specimens were washed with autoclaved deionized water, PBS and immersed in SFM overnight. A metal ring was used to press of each specimen for the complete contact between the plate and the specimens. The MC3T3-E1 cells were trypsinized and counted by a hemacytometer after that the

cells were seeded on the specimen was 40,000 cells/well. The well of TCPS without any specimen was used as a control.

3.3.2.2 Cytotoxicity Evaluation

The neat PCL, BSA-adsorbed PCL, collagen-adsorbed PCL and pre-adsorbed BSA (200, 1500 and 3000 μg/mL of BSA) following collagen-adsorbed (100 μg/mL) were determined for their indirect cytotoxicity and TCPS was used as a control. The specimens were immersed in serum-free medium (SFM) into 24-well plate and incubated at 37°C under 5% CO_2 for 1, 3 and 7 d. 40,000 MC3T3-E1 cells/well were seeded in another 24-well plate for 1 d to allow cells to attach to the plate then starved with SFM for another 1 d. After the starvation, the culture medium was replaced with the prepared extraction medium for 24 hrs. The amount of viable cells was determined in the MTT assay.

3.3.2.3 Cell Attachment and Proliferation

All specimens were immersed in SFM overnight then SFM was removed out. 0.5 mL of 10% MEM was pipetted into each well and 40,000 MC3T3-E1cells were seeded to attach on the surface for 4 hrs, 1 and 3 d. The empty TCPS was used to a control. The plate was incubated at 37°C under 5% CO_2. The viable cell was determine in MTT assay and the cell morphology was observed using SEM.

3.3.2.4 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. The purple formazan product was dissolved in Dimethylsulfoxide (DMSO).

The culture medium was replaced from each well with the MTT solution and incubated at 37°C for 30 min. A buffer solution, containing 900 μL/well of dimethylsulfoxide (DMSO) and 125 μL/well of glycine buffer (pH 10), was added in each well. The solution was shaken for 10 min then determined the viable cells using a microplate reader (Infinite 200 PRO NanoQuant Multimode Microplate Reader) to measure the absorbance at 570 nm. The UV absorbance values

are proportional to the number of cells (MTT assay) that can be converted by calculating standard calibration curves.

3.3.2.5 Cell Morphological Observation

The cell culture medium was removed out and the cell-cultured samples were washed 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. After that, the samples were soaked in hexamethyldisilazane (HMDS) for 5 min and dried in air. The samples were mounted on an SEM stub and coated with thin layer of platinum. The cell morphology of samples was observed at 4 hrs, 1 d and 3 d using a JEOL JSM 5200 scanning electron microscope (SEM).

3.3.2.6 Alkaline Phosphatase (ALP) Activity

MC3T3-E1 cells were cultured on the materials and empty well (TCPS) for 7 days to determine ALP activity. After the removal of the culture medium, each specimen was rinsed with PBS. Alkaline lysis buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton-X 100, pH 10) (100 μL/well) was added, and the specimen was scrapped, and then frozen at -20 °C for at least 30 min. An aqueous solution of 2 mg/mL p-nitrophenyl phosphate (PNPP; Zymed Laboratories) mixed with 0.1 M aminopropanol (10 μL/mL) in 2 mM $MgCl_2$ (100μL/well). Then, it was incubated at 37 °C for 30 min. A solution of NaOH (50 nM at 0.9 mL/well) was added to stop the reaction, and the extracted solution was measured spectrophotometrically at 410 nm. The amount of ALP was calculated against a standard curve and normalized by the total protein content. For the protein assay, each specimen was treated in the same manner as in the ALP assay up to the point where it was frozen. After freezing, a bicinchoninic acid (BCA; Pierce Biotechnology) solution was added to the specimen and incubated at 37 °C for 30 min. The medium solution was determined spectrophotometrically at 562 nm and the amount of total proteins was calculated against a standard curve.

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).