CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials used for Casting Film Mat and Surface Modification

- Poly(ε-caprolactone) (PCL) (M_n = 80,000 g/mol; Aldrich, USA)
- 1,6-hexamethylenediamine (HMD; Aldrich, USA)
- Chloroform (Sigma, USA)

3.1.2 Material used in the Surface Modification

- Ethanol (J. T. Beaker, USA)
- *N,N'*-disuccinimidyl carbonate (DSC; Aldrich, USA)
- Triethylamine (TEA; Sigma, USA)
- Dimethylsulfoxide (DMSO; Lab-Scan (Asia), Thailand)
- Phosphate buffer saline (PBS)
- Bovine serum albumin (BSA; Sigma, USA)
- Crude bone protein (extracted from pork bone legs)
- Ninhydrin (Aldrich, USA)
- Isopropanol (IPA; Fisher Sciencific (Asia), Thailand)
- 1,4-dioxane (Fisher Sciencific (Asia), Thailand)

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 Medium for MC3T3-E1 cells

Minimum Essential Medium (with Earle's Balanced Salts) (MEM; Hyclone, USA), supplemented by 10% fetal bovine serum (FBS; Sorali, Campo Grande, Brazil), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate,

and amphotericin B (Invitrogen Corp., USA)] was used as culture media for MC3T3-E1.

3.1.3.3 Material for Cell Culture Study

- 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; USB Corporation., USA)
- DMEM without phenol red (GibThai Co.,Ltd.)
- Trypsin-EDTA (GibThai Co.,Ltd.)
- Fetal Bovine Serum (Sorali, Campo Grande, Brazil)
- L-Glutamine (GibThai Co.,Ltd.)
- 50% Glutaraldehyde solution (Sigma, USA)
- Hexamethyl disilazane HMDS; Sigma, USA)
- p-nitrophenylphosphate (PNPP; Zymed Laboratories, USA)
- Bicinchoninic acid protein assay (BCA; Pierce Biotechnology, USA)
- L-ascorbic acid (Sigma, USA)
- β-glycerophosphate (Sigma, USA)
- Cetylpyridinium chloride (Sigma, USA)
- Ethanol (J. T. Beaker, USA)

3.2 Equipments for Characterizing of Materials and Studying of Cell Culture

- UV-Vis spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan) was used for determination of the amount of amino group using ninhydrin method.
- Fourier-transformed infrared spectrometer (FT-IR); attenuated total reflection (ATR-FTIR; Thermo Nicolet Nexus 670; resolution of 4 cm^{-1} and 128 scans)
- Contact angle goniometer (KRUSS Gmbh Germany; Model: DSA10-Mk2 T1C) was used to determine contact angles of a water drop on the surfaces.

- Scanning electron microscope (SEM; A JEOL JSM 5410LV) was used to observe the morphology of polymer films.
- X-ray Photoelectron Spectrometer (XPS; AMICUS; Model: KRATOS; MgKα X-ray generated at 2 mA and 10 kV) was used to estimate the elemental composition and chemical state of the elements on the surface.
- A Thermospectronic Genesis10 UV-visible spectrophotometer was used to measure the absorbance of the solution from the MTT

3.3 Methodology

3.3.1 Preparation of Polycaprolactone Film Mats

Polycaprolactone (PCL) film was prepared by dissolving PCL pellets 0.3 g in 5 mL chloroform and casting into a glass Petri dish (diameter 9.6 cm). The solvent was evaporated at room temperature for overnight, and further dried under vacuum for another 24 h at 30°C. Finally, the translucent 6% w/v of PCL films were obtained.

3.3.2 Surface Modification of PCL Film via Aminolysis and further Immobilization of Crude Bone Protein or Bovine Serum Albumin

The film was cut into circle with diameter 1.5 cm and immersed in ethanol/water (1:1 v/v) solution for 2-3 h to clean oily dirt and then washed with a large amount of deionized water. The film was subsequently immersed in 1.5 M 1,6-hexamethylene diamine/isopropanol (IPA) solution for 8 h at 37°C. The resulting aminolyzed PCL film (PCL-NH_2) was rinsed with deionized water for 24 h at room temperature to remove free 1,6-hexamethylenediamine and dried under vacuum at 30°C to constant weight.

Aminolyzed PCL film (PCL-NH_2) was immersed in 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/dimethylsulfoxide (DMSO) solution in the presence of 0.1 M triethylamine for 1 h at ambient temperature followed by rinsing with large amount of deionized water. The film was then directly transferred to 3 mg/mL crude

bone protein/phosphate buffer saline (PBS) solution or 3 mg/mL bovine serum albumin/phosphate buffer saline (PBS) solution at ambient temperature for 24 h. Both of immobilized films were rinsed by soaking in deionized water for 24 h. The modified materials were dried under vacuum before surface characterization.

3.3.3 Preparation of Crude Bone Protein

Crude bone protein (CBP) was extracted from the pork (Tibia) bone. In particular, bone was initially washed and cleaned thoroughly in tap water and then sectioned into small pieces with a high speed motor machine. Pieces of sectioned bones were further crushed into powder in liquid nitrogen. Then, the as-prepared powder was immersed in 1 g/10mL of 0.5 M EDTA at 4°C and shaken continuously on an orbital shaker. After four days, the bony solution was centrifuged and the supernatant was collected, dialyzed for 5 d and lyophilized. The dry CBP was kept in desiccators until use.

3.4 Surface characterization

3.4.1 UV-Vis Spectrophotometer

UV-Vis spectroscopy was used for determination of the amount of amino groups using ninhydrin analysis method. Absorbance in the UV-visible range was measured in a spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). Ninhydrin will react with a free alpha-amino group, NH_2-CH-COOH which is contained in all amino acids, peptides, or proteins, producing Ruhemann's purple colored complex of ninhydrin absorbs the most amount of light at the wavelength of 538 nm.

The ninhydrin analysis method was employed to quantitatively detect the amount of NH_2 groups on the PCL-NH_2 and biomolecule-immobilized PCL films. The film was immersed in 1 M ninhydrin/ethanol solution for 1 h and then was placed into a glass tube at room temperature to evaporate ethanol. After the adsorbed ethanol had evaporated, 1,4-dioxane was added into the tube to dissolve the film When the film surface turned blue, isopropanol (IPA) ,then, was added to

stabilize the blue compound. The absorbance at 538 nm of this mixture was measured on a UV-vis spectrophotometer. A calibration curve was obtained with 1,6-hexamethylenediamine in 1,4-dioxane/IPA (1:1, v/v) solution.

3.4.2 Water Contact Angle Measurements

Contact angle goniometer model 100-00 and a Gilmont syringe with a 24-gauge flat-tipped needle (Ramé-Hart, Inc., USA) were used for the determination of water contact angles dropped on each sample. The measurements were carried out by the sessile drop method in air at room temperature on different areas of sample.

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

All spectra were collected at resolution of 4 cm^{-1} and 128 scan using Nicolet Magna 750 FT-IR spectrometer equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector. A single attenuated total reflection accessory with 45° germanium (Ge) IRE (spectra Tech, USA) and a varible angle reflection accessory (Seagull^{TM}, Harrick Scientific, USA) with a hemispherial Ge IRE were employed for all ATR spectral acquisitions.

3.4.4 Scanning Electron Microscope

The morphology of cells on the materials was examined by using a JEOL JSM 5410L V scanning electron microscope (SEM). Each sample was coated with a thin layer of gold using JEOL- JFC-1100E ion sputtering device prior to SEM observation.

3.4.5 X-ray Photoelectron Spectrometer (XPS)

The XPS experiments were performed using a VG ESCALAB 220i-XL instrument equipped with a monochromatic Al Ka (1486.7 eV photons) and an unmonochromated Mg Ka X-ray source (1253.6 eV photons), a concentric hemispherical analyzer and a magnetic immersion lens (XL lens) to increase the sensitivity of the instrument.

3.5 Biological characterization

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 humidified atmosphere containing 5% CO_2.

3.5.1 Materials Preparation for Cell Seeding and Cell Culturing

Each film mat was cut into circular discs (about 15 mm in diameter) and the disc specimens were placed in wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland), which were later sterilized in 70% ethanol for 30 min. The specimens were then washed with autoclaved de-ionized water, PBS and immersed in SFM overnight. To ensure a complete contact between the specimens and the wells, the specimens were pressed with a metal ring (about 12 mm in diameter). MC3T3-E1 from the culture were trypsinized [0.25% trypsin containing 1 mM EDTA (Invitrogen Crop., USA)] and counted by a hemacytometer (Hausser Scientific, USA) and seeded at a density of about 40,000 cells/cm^2 on the specimens and empty wells of TCPS that were used as control. The culture was maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO_2.

3.5.2 Indirect Cytotoxicity Evaluation

Indirect cytotoxicity test was conducted on TCPS, neat PCL, aminolyzed PCL, crude bone protein- and bovine serum albumin- immobilized PCL. First, the extraction media were prepared by immersing samples 20 mg in 1 ml of a serum-free medium (SFM; containing MEM, 1% L-glutamine, 1 % lactabumin, and 1% antibiotic and antimycotic). Then placed under 5 % CO_2 at 37 °C in 24-well plate for 1 and 7 d. 40,000 MC3T3-E1 cells/well were separately cultured in other 24-well plate to allow cell attach on the plate for 1 d. The cells were further starved with SFM for 1 d. After that, the culture medium was replaced with the extraction

medium. After 24 h cell cultured in extraction medium, MTT assay was carried out to quantify the amount of the viable cells.

3.5.3 Cell Adhesion and Proliferation

The samples were immerged in SFM overnight. After which time, SFM was removed out, then approximately 40,000 MC3T3-E1 cells and 0.5 mL 10% MEM were pipetted into each well containing film mats as well as into the bottom of tissue culture polystyrene plates (TCPS) as a positive control and then incubated under 5 % CO_2 at 37 °C. Cell adhesion was studied on 6 h and 24 h culture period while cell proliferation was investigated on 1, 2 and 3 days culture period. The number of living cells was finally quantified with MTT assay. The morphology of the cell on the materials was observed by SEM.

3.5.4 MTT Assay

MTT assay was used to quantify the number of viable cells, based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of the purple formazan crystal formed is proportional to the number of viable cells. First, culture medium was removed out, replaced by 400 μL of MTT solution (5 mg MTT powder / 100 mL Phenel Red) in each well, and then incubated at 37 °c for 30 min. After incubation, MTT solution was discarded. A buffer solution, containing 900 μL/well dimethylsulfoxide (DMSO) and 125μL/well glycine buffer (pH 10) was added in each well to dissolve the formazan crystal. The solution was shaken for 10 min and then transferred into a cuvette and placed in a spectrophotometer (Thermospectronic Genesis10 UV-visible spectrophotometer) to measure the number of viable cells relative to the absorbance at 540 nm. The numbers of cell were directly indicated to the viable cells.

3.5.5 Morphological Observation of Cultured Cells

After removal of the culture medium, the cell-cultured samples were rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution (diluted from 50 % glutaraldehyde solution with PBS), at 500 µl/well. After 30 min, they were rinsed again with PBS. After cell fixation, the samples were dehydrated in a series of an ethanol solution (i.e. 30, 50, 70, 90, and 100 %, respectively) for about 2 minutes at each concentration. The samples were then let dry in air. After completely dried, the samples were mounted on an SEM stub, coated with gold, and observed by a JEOL JSM 5200 scanning electron microscope (SEM).

3.5.6 Production of Alkaline Phosphatase (ALP) of Cultured Cells

ALP is considered as a relatively early marker of osteoblast differentiation. Cells were allowed to culture on each sample for 3, 5 and 7 days to observe the production of alkaline phosphatase (ALP). The samples were rinsed with PBS after removal of culture medium. 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 samples were 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, USA) mixed with 0.1 M amino propanol (10 µl/well) in 2 mM $MgCl_2$ (100 µl/well) having a pH of 10.5 was prepared and added into the samples. The samples were incubated at 37 °C for 15 minutes. The reaction was stopped by adding 0.9 ml/well of 0.5M NaOH and the extracted solution was transferred to a cuvette and placed in the UV-visible spectrophotometer, from which the absorbance at 410 nm was measured. The amount of ALP was then calculated against a standard curve. In order to calculate for the ALP activity, the amount of ALP had to be normalized by the amount of total protein synthesized. In the protein assay, the samples were treated in the same manner as the ALP assay up to the point was the samples were frozen. After freezing, bicinchoninic acid (BCA; Pierce Biotechnology, USA) solution was added into the samples. The samples were incubated at 37 °C for 15 min. The absorbance of the medium solution was then measured at 562 nm by the UV-vis

spectrophotometer and the amount of the total protein was calculated against a standard curve.

3.5.7 Mineralization

Mineralization refers to cell-mediated deposition of extracellular calcium and phosphorus salts where anionic matrix molecules take up the Ca^{2+}, phosphate ions and serve as nucleation and growth sites leading to calcification. Mineralization was quantified by Alizarin Red-S which is a dye that binds selectively calcium salts and is widely used for mineral staining (the staining product i.e., an Alizarin Red S-calcium chelating product). 40 000 cells of MC3T3-E1 were cultured on each samples for 21 d to observe the production of mineralization. First, cells were cultured in the cultured medium for 3d, then the cultured cells were changed with culture medium in the presence of 50 mg.ml ascorbic acid, 5mM β-glycerophosphate and 25mM dexamethasone. The media was replaced every 2 days. After 21 d, the cells were washed twice with PBS , fixed with cold methanol for 10 minutes, and stained with 1 % Alizarin red solution (prepared in distillated water and adjusted the pH about 4.1 to 4.3 using 10 % ammonium hydroxide) for 3 minutes. After removing the alizarin red-S solution, the cells were rinsed with deionized water and dried at room temperature. The images of each culture were captured and the strain was extracted with 10 % cetylpyridinium chloride in 10 mM sodium phosphate for 20 min and the absorbance of collected dye was read at wavelength 570 nm in spectrophotometer (A Thermo Spectronic Genesis10 UV-visible spectrophotometer). In comparison, tissue culture plate without cell was treated with the procedure as previously described.

3.6 Statistical Analysis

Values are expressed as the mean ± SD. Experiments are performed at least five times and results of representative experiments are presented except where otherwise indicated. Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) with the Least Square Difference (LSD) test multiple

comparisons posttest using SPSS version 11.5 software. The values of $p < 0.05$ were considered statistically significant.