CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials used in the Fibrous Scaffolds Preparation and Surface Modification

- Poly(ε-caprolactone) (PCL) (M_n = 80,000 g/mol; Aldrich, USA)
- Dichloromethane (DCM; Lab-Scan (Asia), Thailand)
- *N,N'*-dimethylformamide (DMF; Lab-Scan (Asia), Thailand)
- 1,6-hexamethylenediamine (HMD; Aldrich, USA)
- *N,N'*-disuccinimidyl carbonate (DSC; Aldrich, USA)
- Ninhydrin (Aldrich, USA)
- Bovine serum albumin (BSA; Sigma, USA)
- Gelatin from porcine skin ,type A (Sigma, USA)
- Gelatin type-B (Sigma, USA)
- Triethylamine (TEA; Sigma, USA)
- Ethanol (J. T. Beaker, USA)
- Isopropanol (IPA; Fisher Sciencific (Asia), Thailand)
- 1,4-dioxane (Fisher Sciencific (Asia), Thailand)
- Dimethylsulfoxide (DMSO; Lab-Scan (Asia), Thailand)
- Phosphate buffer saline (PBS)

3.1.2 Materials used for cell culture

3.1.2.1 Model Cells

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

3.1.2.2 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.

3.1.2.3 Material for Cell Culture Study

- 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; USB Corporation., USA)
- Trypsin-EDTA (GibThai Co.,Ltd.)
- Fetal Bovine Serum (Sorali, Campo Grande, Brazil)
- DMEM without phenol red (GibThai Co.,Ltd.)
- 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)

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-ES30PN/M692 DC
- Syringe with volume size of 25 ml
- Stainless steel needle with gauge number 20 (or the inner diameter of 0.91 mm)
- The rotating drum which covered with aluminum sheet has the width and OD about 14 and 15 cm, respectively.

3.2.2 Equipment for Characterization of Materials

- UV-Vis spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan)
- quartz tube
- 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)
- Scanning electron microscope (SEM; A JEOL JSM 5200)
- X-ray Photoelectron Spectrometer (XPS; Thermo Fisher Scientific Thetaprobe; Monochromatic Al Kα X-ray, the analysis area was approximately 400 μm , while the maximum analysis depth lay in the range of ~4 to 8 nm.)

3.2.3 Equipment for Study of Cell Culture

- A Thermo Spectronic Genesis10 UV-vis spectrophotometer
- Laminar flow hood

3.3 Experimental Procedures

3.3.1 Preparation of Polycaprolactone Scaffolds

3.3.1.1 Preparation and Characterization of Fibrous Scaffolds

Electrospun Polycaprolactone fibrous scaffolds were prepared by electrospinning from a neat 12 % w/v PCL solution in 50:50 v/v DCM/DMF. The as-prepared PCL solution was contained in a glass syringe, the open end of which was connected to a blunt gauge-20 stainless steel hypodermic needle (o.d.0.91 mm) used as the nozzle. An Al sheet wrapped around a rotating cylinder (width and o.d. of the cylinder: 15 cm; rotational speed: 50 rpm) was used as the collector. The distance from the tip of the needle to the surface of the Al sheet defining the collection distance was fixed at 10 cm. A gamma high-voltage research D-ES30PN/M692 power supply was used to generate a high dc potential (i.e., 21 kV). The spinning time was carried out continuously for 10 hours.

Morphological appearance and size of the individual fibers of the scaffolds were examined by a JEOL JSM-5200 scanning electron microscope (SEM). Fibrous scaffolds were dried in vacuum at 30 °C overnight to remove solvent. Each sample was plated in the stub and 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 readings of the fiber diameters from at least five SEM images are statistically analyzed using SemAphore 4.0 software, from which the arithmetic mean values of the individual fibers in the PCL fibrous scaffolds were determined to be 0.93 and 1.26 μm, respectively.

3.3.1.2 Preparation of Crude Bone Protein.

Crude bone protein (CBP) was extracted from the pork 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 0.6 N HCl at 4°C and shaken continuously on an orbital shaker. After three days, the bony solution was centrifuged and the supernatant was collected, dialyzed for 48 h and lyophilized. The dry CBP was kept in desiccators until use.

3.3.1.3 Surface Modification of PCL Scaffold via Aminolysis and Immobilization of Proteins

The scaffold was cut into disc shape with diameter 1.4 cm and immersed in ethanol/water (1:1, v/v) solution for 2 to 3 hours to clean oily dirt and then washed with a large amount of deionized water. The scaffold was subsequently immersed in various concentrations of 1,6-hexamethylene diamine (HMD) /isopropanol (IPA) solution (0.04, 0.06, 0.08, 0.10, 0.20 ,and 0.40 g/ml) for 2 hours at 30 °C. The resulting aminolyzed PCL scaffold was rinsed with deionized water for 24 hours at room temperature to remove free 1,6-hexamethylenediamine and dried under vacuum at 30 °C to constant weight. To determine the optimum concentration of HMD used for aminolysis using ninhydrin method, degreadation experiment, water retention experiment and SEM observation.

Aminolyzed PCL scaffold obtained using the optimum condition was immersed in 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/dimethylsulfoxide (DMSO) solution in the presence of 0.1 M triethylamine (TEA) for 1 hour at ambient temperature followed by rinsing with large amount of deionized water. The scaffold was then directly transferred to 3 mg/ml of gelatin type-A, gelatin type-B, bovine serum albumin (BSA) or crude bone protein (CBP) in phosphate buffer saline (PBS) solution at ambient temperature for 24 hours. PCL scaffolds immobilized with proteins were rinsed by soaking in deionized water for 24 hours. The samples were dried under vacuum before surface characterization.

3.3.2 Characterization of Fibrous Scaffolds

3.3.2.1 Density, Porosity and Pore Volume

The density of the scaffolds ($\rho_{scaffolds}$) can be calculated using the following equation

$$\textbf{Apparent density } (\rho_{scaffold}, \textbf{g/cm}^3) = \frac{m}{txA} \qquad \text{----------(3.1)}$$

where m is the mass of the scaffold (g), t is the thickness of the scaffold (cm) and A is the area of the scaffold (cm^2)

The porosity and pore volume of the scaffolds can be calculated using the following equation (Hou *et al.* 2003).

$$\textbf{Porosity (\%)} = \left(1 - \frac{\rho_{scaffold}}{\rho_{polymer}}\right) x100 \qquad \text{---------(3.2)}$$

$$\textbf{Pore volume} = \left(\frac{1}{\rho_{scaffold}} - \frac{1}{\rho_{polymer}}\right) \qquad \text{----------(3.3)}$$

where $\rho_{scaffold}$ is the apparent density of the fibrous scaffolds(g/cm^3) and $\rho_{polymer}$ is the density of the non-fibrous polymer ($\rho_{polymer}$ of PCL is 1.145 g/cm^3).

3.3.2.2 Water Retention Capacity

The dry scaffolds were weighed and then immersed in 5 mL of 0.1 M PBS solution at 37 °C within 48 hours. At the predetermined time point, scaffold were removed from the solution and carefully placed on glass for 5 seconds to remove the excessive water and weighed immediately. The water retention was calculated by using the following equation (Kothapalli *et al.*, 2005).

$$\textbf{Water Retention (\%)} = \left(\frac{M_{wet} - M_{dry}}{M_{dry}}\right) x100 \text{ ----------(3.4)}$$

where M_{dry} and M_{wet} are the weight of the scaffold before and after immersion in 0.1 M PBS solution respectively. Five measurements were performed for the calculation of an average water retention value.

3.3.2.3 Degradation Study of Fibrous Scaffolds

In degradation study, the disc shape of the fibrous scaffolds with diameter 1.4 cm had been immersed in the 0.1 M PBS pH 7.4 at 37 °C within 48 hours. The fibrous scaffolds were then dried in the vacuum to constant weight.

The rate of degradation can be calculated using the following equation.

$$\textbf{Weight loss (\%)} = \left(\frac{Mf - Mi}{Mi}\right) x100 \text{ ----------(3.5)}$$

where M_i is the initial weight of the scaffolds and M_f and is the weight of the scaffold at the given degradation time point, immersed in 0.1 M PBS solution. Five measurements were performed for the calculation of an average water degradation rate value.

3.3.3 Determination of the Amino Groups on PCL Surface after Aminolysis and Protein Immobilization

The ninhydrin analysis method was carried out to quantitatively determine the amount of NH_2 groups on the aminolysed PCL and biomolecule-

immobilized PCL scaffolds. The scaffold was immersed in 1 M ninhydrin/ethanol solution for 15 minutes. After the adsorbed ethanol had evaporated, 1,4-dioxane was added to dissolve the scaffold. When the scaffold surface turned blue, isopropanol (IPA) was then added to stabilize the blue compound. This mixture was transfered to quartz tube and measured the absorbance at the wavelength of 538 nm using a UV-vis spectrophotometer. A calibration curve was obtained with known concentration of 1,6-hexamethylenediamine in 1,4-dioxane/IPA (1:1, v/v) solution.

3.3.4 Surface Characterization

3.3.4.1 Water Contact Angle Measurements

Contact angle goniometer (KRUSS Gmbh Germany; Model: DSA10-Mk2 T1C) were used for the determination of water contact angles. The measurements were carried out by the sessile drop method in air at room temperature. The reported angle was an average of 5 measurements on different area of each sample.

3.3.4.2 UV-Vis Spectrophotometer

UV-vis spectrophotometer Model Shimadzu, UV-1800 was used for determination of the amount of amino group using ninhydrin method on the modified PCL surface. Ninhydrin will react with a free alpha-amino group, NH_2-C- which is contained in all amino acids, peptides, or proteins, producing Ruhemann's purple colored complex of ninhydrin absorbs the visible light at the wavelength of 538 nm.

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

All spectra are collected at resolution of 4 cm^{-1} and 128 scans 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™, Harrick Scientific, USA) with a hemispherial Ge IRE are employed for all ATR spectral acquisitions. Chemical functional groups that were present on the surfaces of both the neat and the surface-modified fibrous scaffolds were analyzed by ATR-FTIR spectrometer.

3.3.4.4 X-ray Photoelectron Spectrometer (XPS)

XPS was used to estimate the elemental composition and chemical state of the elements on the surface. The analysis of the samples was carried out using a Thermo Fisher Scientific Thetaprobe XPS. Monochromatic Al K_α X-ray was employed for analysis of one spot on each sample with photoelectron take-off angle of 50° (with respect to surface plane). The analysis area was approximately 400 μm, while the maximum analysis depth lay in the range of ~4 to 8 nm. A special designed electron flood gun with a few eV Ar^+ ion was used for the charge compensation. Electron beam and ion beam were focused and steered towards the analysis position. Further correction was made based on adventitious C 1s at 285.0 eV using the manufacturer's standard software. Survey spectra were acquired for surface composition analysis with Scofield sensitivity factors.

3.3.4.5 Scanning Electron Microscope

The morphology of cells on the materials was examined by using a JEOL JSM 5200 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.

3.3.5 Cell Culture Studies

Osteoblast (MC3T3-E1) cell lines are used. MC3T3-E1 cells are 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.

3.3.5.1 Cytotoxicity Evaluation

3.3.5.1.1 Direct Cytotoxicity

This test was used to evaluate the potential for use of protein directly to the cell. 4×10^4 MC3T3-E1 cells/well were cultured in 24-well plate to allow cell attachment on the plate. After incubation under 5 % CO_2 at 37 °C at least 4 hours, the cells were starved with a 2 % culture media (2 % MEM; containing MEM, 2 % FBS, 1 % L-glutamine, 1 % lactabumin, and 1 % antibiotic

and antimycotic formulation.) for 24 hours. After starvation, the culture medium was removed and replaced with the as-prepared extraction media (2 % culture media with appropriate amount of proteins ; gelatin type-A , gelatin type-B, bovine serum albumin, and crude bone protein) and later incubated for 1, 2, and 3 days. The number of living cells was finally quantified with MTT assay.

3.3.5.1.2 Indirect Cytotoxicity

The indirect cytotoxicity evaluation was conducted on modified and unmodified PCL scaffolds in adaptation from the ISO10993-5 standard test method . The samples are pre-washed with 70 % ethanol in water for 30 minutes, washed twice with PBS solution and deionized water and immerged in a 2% MEM culture medium (2 % MEM; containing MEM, 2 % FBS, 1 % L-glutamine, 1 % lactabumin, and 1 % antibiotic and antimycotic formulation.) under 5 % CO_2 at 37 °C in 24-well plate for 24 hours to prepare the extraction media. 4×10^4 MC3T3-E1 cells/well were separately cultured in other 24-well plate to allow cell attachment on the plate at least 4 hours. The cells were further starved with a 2 % MEM and replaced in 2 hours for 3 times. After incubation under 5 % CO_2 at 37 °C, the culture medium was removed and replaced with the as-prepared extraction media and later incubated for another 1, 2 and 3 days. The number of living cells was finally quantified with MTT assay.

3.3.5.2 Cell Adhesion and Proliferation

Both modified and unmodified PCL scaffolds (diameter 1.4 cm) are sterilized by soaking in 70 % ethanol in water for 30 minutes, washed twice with PBS solution and deionized water and immerged in a 10 % MEM culture medium under 5 % CO_2 at 37 °C in 24-well plate for 24 hours. After removing of culture media, approximately 4×10^4 MC3T3-E1 cells and 0.5 mL culture medium were pipetted into each well containing as-prepared scaffolds as well as into the bottom of tissue culture polystyrene plates (TCPS) as a control and then incubated under 5 % CO_2 at 37 °C. Cell adhesion was studied on 2, 4, 6 hours 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.

3.3.5.3 MTT Assay

The MTT assay is 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. After desirable culture period, the culture medium was removed to discard the unattached cell and incubated at 37 °C for 30 minutes with 300 µl/well of MTT solution at 0.5 mg/ml culture medium without phenol red. After incubation, MTT solution was removed. A buffer solution containing dimethylsulfoxide (DMSO : 900 µl/well) and glycine buffer (pH = 10 : 125 µl/well) was added into the wells to dissolve the formazan crystals. After 10 minutes of rotary agitation, the solutions were then transferred into a cuvette and placed in a spectrophotometer (Thermospectronic Genesis10 UV-visible spectrophotometer) to measure the number of viable cells at absorbance 540 nm .

3.3.5.4 Morphological Observation of Cultured Cells

After removal of the culture medium, the cell-cultured scaffold 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 minutes, they were rinsed again with PBS. After cell fixation, the samples were dehydrated in an ethanol solution of varying concentration (i.e. 30, 50, 70, 90, and 100 %, respectively) for about 2 minutes at each concentration. The samples were then dried in 100 % hexamethyldisilazane (HMDS) for 5 minutes and later let dry in air after removal of HMDS. 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).

3.3.5.5 Production of Alkaline Phosphatase of Cultured Cells

ALP is considered as a relatively early marker of osteoblast differentiation. Cells were cultured on scaffold samples for 3, 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 minutes prior to the next step. An aqueous solution of 2 mg/ml *p*-nitrophenyl phosphate 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 50 mM 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 minutes. 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.

3.3.5.6 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). MC3T3-E1 cells (4×10^4 cells/well in 24-well plate) were cultured on scaffold samples for 16 days to observe the production of mineralization. The isolated cells were plated in 24-well plate and cultured in the cultured medium. The cultured cells were changed after a 24 hours attachment period with culture medium in the presence of 5 mM *β*-glycerophosphate and 50 µg/ml ascorbic acid. The media was replaced every 2 days thereafter. After 16, 21 and 30 days, respectively, 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 5 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 1 hour 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.

3.3.5.7 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 17 software. $p < 0.05$ or $p < 0.01$ is considered statistically significant.