CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials Used in the Fabrication of the Fibrous Scaffolds

- Poly(lactic acid) (PLA) (M_n = 80,000 g/mol; Aldrich, USA)
- Dichloromethane (Carlo Erba, Italy)
- *N,N'*-dimethylformamide (DMF; Lab-Scan (Asia), Thailand)

3.1.2 Materials Used in the Surface Modification

- 1,6-hexamethylenediamine (HMD; Aldrich, USA)
- *N,N'*-disuccinimidylcarbonate (DSC; Aldrich, USA)
- Ninhydrin (Aldrich,USA)
- Type-I collagen (calf skin; Sigma, USA)
- Triethylamine (TEA; Sigma, USA)
- Ethanol (Lab-Scan (Asia), Thailand)
- Isopropanol (IPA, Lab-Scan (Asia), Thailand)
- 1,4-dioxane (Lab-Scan (Asia), Thailand)
- Dimethylsulfoxide (DMSO; Lab-Scan (Asia), Thailand)
- 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 fibroblasts (L929) and pre-osteoblastic cells (MC3T3-E1).

3.1.3.2 Medium for L929 Culture

Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS; BIOCHROM AG), 1% L-glutamine (Invitrogen Corp.), and a 1% antibiotic and antimycotic

formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)] was used as culture media for L929.

3.1.3.3 Medium for MC3T3-E1 Culture

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

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 was used to generate positive DC voltage
- Syringe with volume size of 25 ml was served as a container for polymer solutions.
- Stainless steel needle with gauge number 20 (or the inner diameter of 0.91 mm) was used as the electrode to conduct the electrical from power supply to the solutions.
- The rotating drum which covered with aluminum sheet was used as a collector. Width and OD of the rotating drum were 14 and 15 cm, respectively.
- A Kd Scientific syringe pump was used to control the feed rate of the polymer solution at about 1 ml/h.

Figure 3.1 show the electrospinning apparatus.

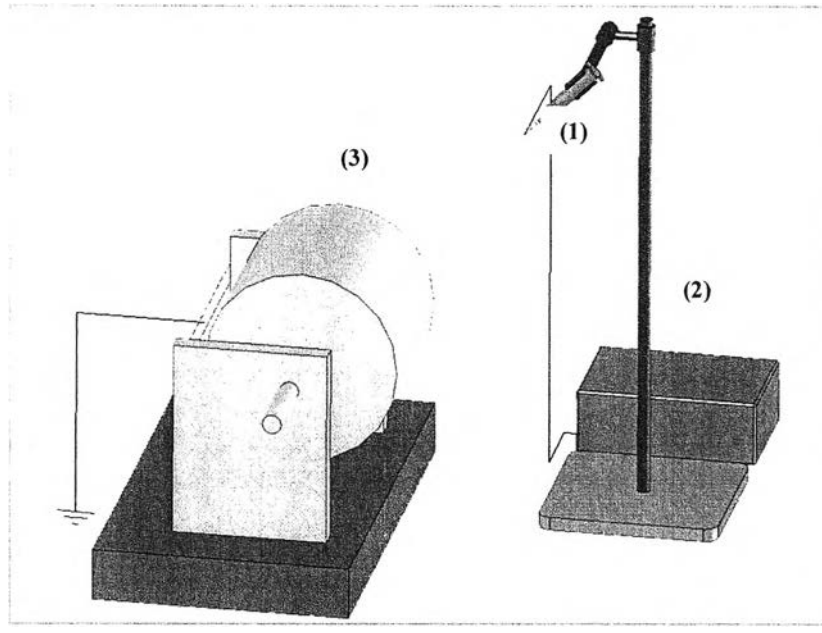

Figure 3.1 Schematic of a typical electrospinning apparatus, including: (1) syringe needle, (2) voltage supply, and (3) collector.

3.2.2 Equipment for Characterization of Materials

- 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 electrospun fibers.
- 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.

3.2.3 Equipment for Study of Cell Culture

- 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 Poly(lactic acid) (PLA) Electrospun Fiber Mats

1. The spinning solution was prepared by dissolving the polymer, PLA pellet in 70:30(v/v) DCM/DMF to obtain 10% PLA solution.
2. The as-prepared solution was continuously stirred until clear solution was obtained.
3. The solution was fed into a glass syringe fitted with a needle.
4. The needle was connected to the emitting electrode of positive polarity from a voltage dc power supply. Applied electrical and collection distance were set at 20 kV and 18 cm, respectively
5. The as-spun fibers were collected on an aluminum sheet wrapped around a home-made rotating cylinder (width and diameter =15 cm).
6. The obtained fibrous membranes were dried in *vacuo* at room temperature prior to further modification.

The morphological appearance of the as-spun fibers was observed by a JEOL JSM-5200 scanning electron microscope (SEM). 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 of nanofibers at 100 different points in the SEM images of 10,000x magnification with a SemAphore 4.0 Software. The diameters were presented as the average ± standard deviation.

3.3.2 Surface Modification of PLA Fibrous Scaffolds via Aminolysis and Type-I Collagen Immobilization

1. The PLA fibrous membranes were first immersed in an ethanolic aqueous solution (1:1 v/v) for 2 to 3 h to clean the fiber surfaces.

2. Washed with a large quantity of deionized water
3. The fiber mats were subjected to aminolysis through an immersion in a HMD/IPA solution of varying concentration (i.e., 0.02, 0.04,.., 0.1 gml^{-1}) and varying reaction time (2, 5, 8, 15, 20, 30 min) at 50°c.
4. The aminolyzed fibrous scaffolds were then rinsed with deionized water for 24 h at room temperature to remove unreacted HMD.
5. Dried *in vacuo* at room temperature to reach a constant weight
6. Immobilization of type-I collagen on the surface of the fibrous scaffolds was carried out by first activating the aminolyzed PLA fibrous scaffolds in 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/ dimethylsulfoxide (DMSO) solution in the presence of 0.1 M triethylamine for 3 h at room temperature.
7. Rinsed the fiber mats with a large quantity of deionized water
8. The activated PLA fibrous scaffolds were then immersed in 0.5 or 3 $mg{\cdot}ml^{-1}$ of collagen/phosphate buffer saline (PBS) solution at room temperature for 24 h.
9. The collagen-immobilized PLA fibrous scaffolds were rinsed by soaking in a large quantity of deionized water for 24 h.
10. Dried *in vacuo* at room temperature

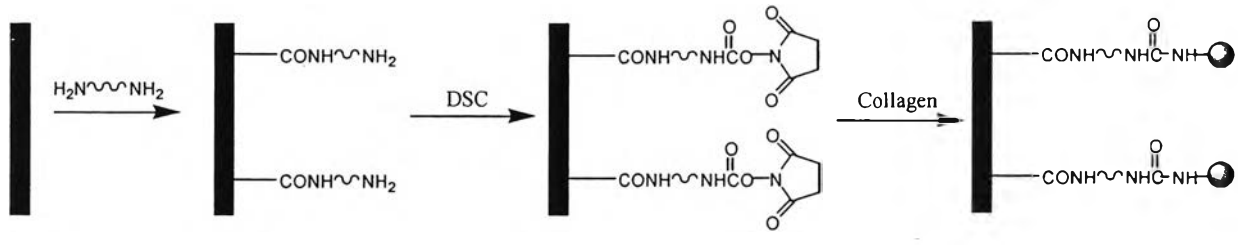

Figure 3.2 The chemical pathway for the immobilization of collagen.

3.4 Surface Characterizations

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-C-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 570 nm.

Procedure

1. The fibrous scaffolds were immersed in 1 M ninhydrin/ethanol solution for 1 min in a glass tube.
2. Heating at 80°C for 15 min to accelerate the reaction between ninhydrin and the NH_2 groups (the surfaces of the scaffolds would turn blue)
3. After complete evaporation of absorbed ethanol, 1,4-dioxane was added in the tube to dissolve the scaffolds.
4. IPA was then added to stabilize the blue compound.
5. The amount of NH_2 groups was finally quantified by observing the absorbance of the obtained mixture at 538 nm using a UV-Vis spectrophotometer against a predetermined calibration curve that was obtained from HMD solutions in 1,4-dioxane/IPA (1:1 v/v)

3.4.2 Water Contact Angle Measurements

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 PLA fibrous scaffolds. The measurements were carried out by the sessile drop method in air at room temperature in pentuplicate on different areas of each sample.

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

Chemical functional groups that were present on the surfaces of both the neat and the surface-modified fibrous scaffolds were analyzed by Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR; Thermo Nicolet Nexus 670) at a resolution of 4 cm^{-1} and 128 scans.

3.4.4 X-ray Photoelectron Spectrometer (XPS)

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.

3.5 Biological characterizations

The study of *in vitro* pre-osteoblastic (MC3T3-E1) cell response to the e-spun scaffolds was conducted on TCPS, aminolyzed PLA, activated PLA, and collagen-immobilized PLA.

3.5.1 Cytotoxicity of the E-spun Scaffolds

Two types of cells were used: 1) pre-osteoblastic cells (MC3T3-E1) and 2) mouse fibroblasts (L929). Indirect cytotoxicity test was conducted on TCPS, aminolyzed PLA, activated PLA, collagen-immobilized PLA, and polycaprolactone/hydroxyapatite (PCL/HA; as positive control). First, extraction media were prepared by immersing samples (about 15 mm in diameter) in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1 % lactabumin, and 1% antibiotic and antimycotic formulation for L929 and containing MEM, 1% L-glutamine, 1 % lactabumin, and 1% antibiotic and antimycotic formulation for MC3T3-E1) for 1, 3, and 7 d. Each of these extraction media was used to evaluate the cytotoxicity of the scaffolds. L929 and MC3T3-E1 were separately cultured in wells of a 24-well culture plate in 10% serum-containing DMEM and MEM, respectively, for 16 h to allow cell attachment on the plate. Then, the cells were

starved with SFM for 24 h, after which time the medium were replaced with an extraction medium. After 24 h of cell culturing in the extraction medium, MTT assay was carried out to quantify the amount of viable cells.

Another experiment was conducted to confirm the cytocompatibility of the materials on MC3T3-E1. Extraction media were prepared by immersing samples (about 15 mm in diameter) in a serum-free medium (SFM; MEM, 1% L-glutamine, 1 % lactabumin, and 1% antibiotic and antimycotic formulation for MC3T3-E1) for 7 d. The samples were treated in the same manner as the previous experiment up to the point was the cells were starved. After that the SFM was replaced with 2% serum-containing MEM diluted with as-prepared extraction medium for 1, 2, and 3 d to allow cell growth on the plate.

3.5.2 Quantification of Viable Cells (MTT Assay)

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (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 purple formazan crystals formed is proportional to the number of viable cells. First, each culture medium was incubated at 37°C for 15 min with 300 μl/well of MTT solution at 0.5 mg/ml without phenol red. After incubation, MTT solution was removed. A buffer solution containing dimethylsulfoxide (DMSO; Carlo Erba, Italy) (900 μl/well) and glycine buffer (pH = 10) (125 μl/well) was added into the wells to dissolve the formazan crystals. After 10 min of rotary agitation, the solutions were then transferred into a cuvette and placed in a Thermospectronic Genesis10 UV-visible spectrophotometer, from which the absorbance at 540 nm representing the number of viable cells was measured.

3.5.3 Cell Culturing and Cell Seeding

Mouse fibroblasts (L929) and mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were used as reference cell lines. L929 and MC3T3-E1 were cultured as monolayer in 10% serum-containing DMEM and MEM, respectively. The medium was replaced every 2 days and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Each scaffold 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 subsequently immersed in MEM 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). For attachment and proliferation, MC3T3-E1 were seeded at a density of about 60,000 cells/cm^2 and 30,000 cells/cm^2 on the scaffold specimens and empty wells of TCPS that were used as control, respectively. For indirect cytotoxicity and alkaline phosphatase activity evaluations, MC3T3-E1 were seeded at a density of about 40,000 cells/cm^2 on the scaffold specimens and empty wells of TCPS. The culture was maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO_2.

3.5.4 Cell Attachment and Proliferation

For attachment study, MC3T3-E1 was allowed to attach to TCPS, neat PLA, aminolyzed PLA, activated PLA, collagen-immobilized PLA fibrous scaffolds for 2, 4 and 16 h, respectively. At each specified seeding time, the number of the attached cells was quantified by MTT assay. Each sample was rinsed with PBS to remove unattached cells prior to MTT assay. For proliferation study, the cells were first allowed to attach on the specimens for 16 h. The proliferation of cells on the specimens was determined after 1, 2, and 3 days, respectively. The number of cells was, again, quantified by MTT assay. Morphological appearance of the cells at 2 and 16 h cell culturing was observed by SEM.

3.5.5 Morphological Observation of Cultured Cells

After removal of the culture medium, the cell-cultured scaffold specimens were rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution, which was diluted from 50% glutaraldehyde solution

(Sigma, USA) with PBS, at 500 µl/well. After 30 min, they were rinsed again with PBS. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e. 30, 50, 70, 90, and 100%, respectively) for about 2 min at each concentration. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and later let dry in air after removal of HMDS. After completely dried, the specimens 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 of Cultured Cells

Cells were cultured on scaffold specimens for 3, 5, and 7 days to observe the production of alkaline phosphatase (ALP). The specimens were rinsed with PBS after removal of culture medium. Alkaline lysis buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton-X100, pH 10) (100 µl/well) was added and the samples were scrapped and then frozen at -20°C for at least 30 min prior to the next step. 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 specimens. The specimens were incubated at 37°C for 15 min. 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 specimens were frozen. After freezing, bicinchoninic acid (BCA; Pierce Biotechnology, USA) solution was added into the specimens. The specimens were incubated at 37°C for 15 min. The absorbance of the medium solution was then measured at 562 nm by the UV-visible spectrophotometer and the amount of the total protein was calculated against a standard curve.

3.6 Statistical analysis

Values expressed as the mean ± standard deviation. Statistical analysis of different data groups was performed using One-Way Analysis of Variance (ANOVA) with the least-significant difference (LSD) test using SPSS software version 11.5. The values of p lower 0.05 were considered statistically significant.