CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Polymers

- Poly(caprolactone) (PCL, M_w = 80 000 g/mol; Aldrich, USA)
- Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV, Mw = 680 000 g/mol; Aldrich.USA)

3.1.2 Solvents

- Chloroform (Sigma,USA)
- Dimethylsulfoxide (DMSO; Lab-Scan (Asia), Thailand)
- Phosphate buffer saline (PBS)
- Bovine serum albumin (BSA; Sigma, USA)
- 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazoliun bromide (MTT; Sigma, USA)

3.2 Equipment

- Petri Dish (10 cm in diameter)
- Differential scanning calorimeter (Perkin-Elmer; Model: DSC7) was used to determine misibility of the blended films.
- Contact angle goniometer (KRUSS Gmbh; Model: DSA10-Mk2T1C; Germany) was used for the measurement of water contact angles on the surfaces.
- Attenuated total reflectance-Fourier transform infrared spectrometer (ATR-FTIR; Thermo Nicolet; model: Nexus 670) was used to determine functional groups on the surfaces

- Scanning electron microscope (SEM; Hitachi; model: FE-SEM S4800) was used to investigate surface topography of the film.
- X-Ray Diffraction (XRD; Bruker AXS , Model:D8 Advance, Germany) was used to determine degree of crystallinity.
- Atomic Force Microscope (AFM; PARK; Model: XE-100) was used to examine the topography of membrane.
- Fluorescence microscope (ZEISS; Model: Axio Observer.Z1, Germany) was used to investigate actin staining on the surface of the films.

3.3 Methodology

3.3.1 Preparation of Film Mats

Blended film was prepared by dissolving PCL pellets and PHBV powders by varying PHBV contents (Table 3.1) 0.3 g in 5 mL chloroform at 80°C and casting into a glass Petri dish (diameter 10 cm). The solvent was evaporated at room temperature for overnight, and further dried under vacuum for another 24 hours at 30°C. Finally, the 6% w/v of blended PCL/PHBV films were obtained.

Table 3.1 Blending compositions of PCL and PHBV

PCL	PHBV
100	0
90	10
80	20
70	30
60	40
50	50
0	100

3.3.2 Coating Films Mats with Protein

The film was immersed in 3 mg/mL bovine serum albumin/phosphate buffer saline (PBS) solution at ambient temperature for 24 hours and then the films were rinsed by soaking in deionized water for 24 hours. The materials were dried under vacuum before surface characterization.

3.4 Surface Characterizations

3.4.1 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 blended films. The measurements were carried out by the sessile drop method in air at room temperature in pentuplicate on different areas of each sample.

3.4.2 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 variable angle reflection accessory (SeagullTM, Harrick Scientific, USA) with a hemispherical Ge IRE were employed for all the ATR spectral acquisitions.

3.4.3 Scanning Electron Microscope (SEM)

The morphology of cells on the materials was examined by using a Hitachi FE-SEM S4800 scanning electron microscope (SEM). Each sample was coated with a thin layer of platinum using Hitachi- E-1010 ion sputtering device prior to SEM observation.

3.4.4 Differential Scanning Calorimeter (DSC)

Differential Scanning Calorimetry (Perkin-Elmer; DSC7) was used to analyze the thermal properties of the solvent-cast polymer films. Samples were sealed hermetically in aluminium pans and heating was carried out in the range of 0°C to 220°C with a heating rate of 10°C/minute.

3.4.5 Atomic Force Microscope (AFM)

Atomic Force Microscope (AFM; PARK; Model: XE-100) was used to investigate roughness of the surface. The images were made in IC-AFM mode. The measurements were performed at 25°C at 30-40% relative humidity in air.

There are a number of amplitude parameters, for example Ra which also known as centre line average; the arithmetic average height parameter, Rq or root mean square; the standard deviation of the z height values, and Rz or the difference in height between the average of the five highest peaks, and the five lowest valleys along the assessment length of the profile (Whitehead and Verran, 2006).

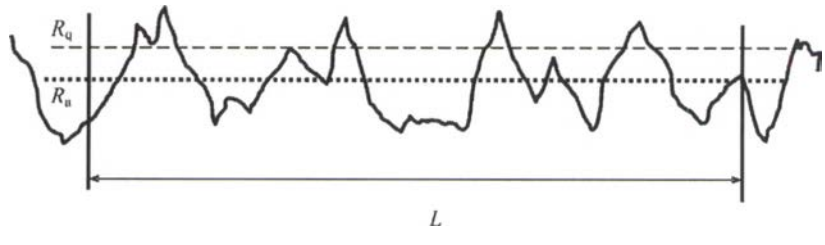

Figure 3.1 Schematic derivation of R_a and R_q surface roughness values.

$$R_a = \frac{1}{L}\sum_{n=1}^{N} r_n$$

$$R_q = \sqrt{\frac{1}{N}\sum_{n=1}^{N} r_n^2}$$

$$R_z = \frac{1}{n}\left(\sum_{i=1}^{n} p_i - \sum_{i=1}^{n} v_i\right)$$

3.4.6 X-ray Diffraction Techniques (XRD)

X-ray diffraction (XRD) was conducted with a D8 Advance (Bruker AXS , Germany) at a scan speed of 1°/min. The diffraction intensity of Cu Kα radiation was under the condition of 40 kV and 30 mA with a wave length of 0.15406 nm. The scan scope was between 5° and 50°. The degree of crystallinity (X_c) was calculated by (Zhang *et al.*, 2010)

$$Degree\ of\ crystallinity = \frac{F_c}{F_t} = \frac{F_c}{F_c + F_a}$$

Where F_c is the crystalline area

F_a is the amorphous area

F_t is the total area

3.5 Protein Adsorption

The films were placed in the protein solution; bovine serum albumin in PBS (3 mg/ml), for 24 hours. After immersing, the films were washed the excess protein with deionize water, then dried them in the oven at 35°C for 24 hours. The remaining proteins (adsorbed) on the films were measure by incubation in 1% sodium dodecyl sulfate

(SDS) solution for 30 minutes. The total amount of protein was measured using BCA rotein Passay (Pierce, Rockford, IL).

The BCA™ Protein Assay is a detergent-compatible fomulation on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein by using Microplate reader (TECAN; Model: Infinite M200). This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimeteric detection of the cuprous cation(Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentration over a broad working range.

Procedure

1. Pipette 25 µl of samples (SDS solution with protein) replicate into a microplate well
2. Add 200 µl of the BCA™ working reagent(WR) to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 60°C for 30 minutes.
4. Measure the absorbance at 562 nm on a plate reader

3.6 Biological Characterizations

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.

3.6.1 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 minutes. 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 glass ring (about 12 mm in diameter). MC3T3-E1 from the culture were trypsinized [0.25% trypsin containing 1 m MEDTA (Invitrogen Crop., USA)] and counted by a hemacytometer (Hausser Scientific, USA). To characterize the cell behavior in each stage, MC3T3-E1 were 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.

3.6.2 Indirect Cytotoxicity Evaluation

Indirect cytotoxicity test was conducted on TCPS, PCL, PHBV, and PCL/PHBV blended films. First, the extraction media was prepared by immersing samples in 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, 3 and 7 days. 40,000 MC3T3-E1 cells/well were separately cultured in other 24-well plate to allow cell attach on the plate for 1 day. The cells were further starved with SFM for 1 day. After that, the culture medium was replaced with the extraction medium. After 24 hour cell cultured in extraction medium, MTT assay was carried out to quantify the amount of the viable cells.

3.6.3 Cell Attachment and Cell Proliferation Study

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 as-prepared films 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 4 hours and 16 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. The morphology of the cell behaved on the materials was observed by SEM.

3.6.4 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 in each well and incubated in 0.4 mL MTT solution at 37 °C for 30 minutes. After incubation, MTT solution was remove. 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 at absorbance 540 nm.

3.6.5 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 minutes, 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 to dry in air. After completely dried, the samples were mounted on an SEM stub, coated with platinum, and observed by a Hitachi FE-SEM S4800 scanning electron microscope (SEM).

3.6.6 Actin Staining

Staining of actin was performed after 4 hours and 1 day of culture. Cells were washed with PBS and then fixed with 4% paraformaldehyde for 30 minutes. After 3 washings (10 minutes each in PBS), Actin was stained with Phalloidine-rhodamine for 60 minutes at room temperature and rinsed 3 times (10 minutes each in PBS). Samples were put on the glass slide with and viewed on a Fluorescence microscope.

3.6.7 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). 40000 cells of MC3T3-E1 were cultured on each samples for 21 days to observe the production of mineralization. First, cells were cultured in the cultured medium for 3 days, then the cultured cells were changed with culture medium in the presence of 50μL β-glycerophosphate, 50 μL ascorbic acid and 500 μL dexamethazone. The media was replaced every 2 days. After 21 days, 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 stain was extracted with 10 % cetylpyridinium chloride in 10 mM sodium phosphate for 20 minutes 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.

3.7 Statistical Analysis

Values are expressed as the mean ± Standard Deviation. Experiments are performed at least three times and the significance among three data sets was determined by one-way ANOVA analysis using Scheffe's test. The statistical significance was accepted at $p < 0.05$.