CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Raw Silk Fibers and Shrimp Shells

The raw silk fibers of *Bombyx mori* were obtained from Queen Sirikit Sericulture Center (Thailand). The shells of *Penaeus merguiensis* shrimps were kindly provided by Surapon Foods Public Co., Ltd. (Thailand).

3.1.2 Rhamnolipid-producing microorganisms

Pseudomonas aeruginosa SP4 was isolated from petroleum contaminated soil in Thailand (Paisanjit, 2006).

3.1.3 Other Chemicals

Pluronic F-68 and 1-[4-(phenylazo)phenylazo]-2-naphthol, or Sudan III, (90% dye content), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (USA). Sodium dodecyl sulfate (SDS) (96% purity) was supplied by Ajax Finechem (Australia). Cholesterol (≥95% purity) was provided by Fluka. Acetonitrile (HPLC grade), sodium chloride (NaCl) (99.0% purity), and ethanol (C_2H_5OH) (99.8% purity) were purchased from Labscan Asia Co., Ltd. (Thailand). All chemicals were used as received without further purification.

3.2 Equipments

3.2.1 High Performance Liquid Chromatograph

The key components in the biosurfactant extracted from the liquid culture of *P. aeruginosa* SP4 were fractionated using a high performance liquid chromatograph (HPLC) (an Alltech 580 autosampler, an Alltech HPLC pump, model 626, and an

Inertsil® ODS-3 column) equipped with an evaporative light scattering detector (ELSD) (Alltech, 2000ES).

3.2.2 Fourier Transform Infrared Spectrophotometer

A Thermo Nicolet Nexus 670 Fourier transform infrared (FT-IR) spectrometer equipped with an attenuated total reflectance (ATR) crystal accessory was used to provide a chemical analysis of the components in the biosurfactant produced by *P. aeruginosa* SP4.

3.2.3 Nuclear Magnetic Resonance Spectrometer

The ^{1}H nuclear magnetic resonance (NMR) spectra of each component in the biosurfactant product were achieved from an FT-NMR 500 mHz spectrometer (JEOL, JNM-A500).

3.2.4 Mass Spectrometer

A Waters mass spectrometer was used to determine a molecular weight of each component in the biosurfactant produced by *P. aeruginosa* SP4.

3.2.5 Tensiometer and Drop Shape Analysis System

The surface tension of the aqueous solution at different surfactant concentrations was measured with either du Nöuy ring-type tensiometer (Krüss, K10T) or drop shape analysis system (Krüss, DSA10 Mk2) using the pendant drop technique. The same drop shape analysis system (Krüss, DSA10 Mk2) was also employed to determine the contact angles formed between the water drops and the studied surfaces by using the sessile drop technique.

3.2.6 UV/Vis spectrophotometer

A UV/Vis spectrophotometer (Shimadzu, UV-2550) was used to measure the turbidity of the biosurfactant solutions and the Sudan III concentration in the sample.

3.2.7 Brookhaven ZetaPALS Instrument

A Brookhaven ZetaPALS instrument was used in the conductivity, dynamic light scattering (DLS), and zeta potential measurements.

3.2.8 Transmission Electron Microscopy

The morphology of the biosurfactant microstructures was observed with a transmission electron microscope (JEOL, JEM-2100).

3.2.9 Quartz Crystal Microbalance Instrument

A quartz crystal microbalance instrument (a Q-sense E1 system) was used to quantify the amount of the biosurfactant adsorbed onto the polymeric substrate.

3.2.10 Surface Plasmon Resonance Instrument

The adsorption behavior of the biosurfactant onto the polymeric substrate was studied by using a surface plasmon resonance instrument (AUTOLAB, ESPRIT).

3.2.11 Surface Probe Microscopy

The surface topography of the polymeric substrates was investigated by using a scanning probe microscope (SPM) (Veeco, Nanoscope IV).

3.2.12 Microplate Reader

The fluorescent signal from the cell culture sample was measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA).

3.3 Methodology

3.3.1 Preparation of Regenerated Silk Fibroin

The silk fibroin solution was prepared according to the work of Wongpanit *et al.* (2007). The raw silk fibers of *B. mori* were first boiled in a 0.05%

(w/v) Na_2CO_3 solution for 15 min, followed by a thorough rinse with hot water. The boiling step, or the degumming process, was repeated two times to remove silk sericin, a family of glue-like proteins in the raw silk fibers. The degummed silks were dried at 40°C overnight before dissolving in a solvent having a 1:2:8 $CaCl_2$-to-C_2H_5OH-to-H_2O molar ratio at 78°C. The solution was dialyzed against distilled water for 4 days with a daily change of dialyzed media. The dialyzed solution was centrifuged at 10,000 rpm for 10 min before holding at 4°C. The concentration of the as-prepared silk fibroin solution was about 4.5% (w/v) before being diluted to the desired concentration.

3.3.2 Preparation of Chitosan

The preparation of chitosan from the shells of *P. merguiensis* shrimps was previously described (Thanpitcha *et al.*, 2006). Briefly, the shrimp shells were cleaned and then dried under sunlight before grinding into small pieces. The grinded shrimp shells were immersed in a 1 M HCl solution for 2 days with occasional stirring and were washed with distilled water until becoming neutral. The demineralized shrimp shell chips were soaked in a 4% (w/v) NaOH solution at 80–90°C for 4 h, followed by an excessive wash with distilled water. The obtained product, or chitin, was used to prepare chitosan by heating in a 50% (w/v) NaOH solution at 110°C for 1 h using an autoclave. After the deacetylation reaction, the chitosan platelets was washed with distilled water until becoming neutral and were dried at 60°C for 24 h. The viscosity-averaged molecular weight of the chitosan product calculated from the Mark-Houwink equation (Wang *et al.*, 1991) was about 1.1×10^6 Da. The degree of deacetylation (%DD), determined from the Fourier transform infrared (FT-IR) spectroscopy following the method of Baxter *et al.* (1992), was 85.0%.

3.3.3 Production and Extraction of Biosurfactant

To produce biosurfactant, an inoculum was prepared by transferring the bacterial colonies into a nutrient broth, and the culture was incubated at 37°C in a shaking incubator at 200 rpm for 22 h. Then a nutrient broth containing 2% inoculum

and 2% palm oil was incubated at 37°C under aerobic condition in a shaking incubator at 200 rpm for 48 h to obtain the highest microbial and surfactant concentration (Paisanjit, 2006). After that, the solution was centrifuged at 4°C and 8500 rpm for 20 min to remove the bacterial cells. The obtained supernatant was further treated by acidification to pH 2.0 using 6 M HCl solution, and the acidified supernatant was left overnight at 4°C for the complete precipitation of the biosurfactants (Yakimov *et al.*, 1995). After centrifugation, the precipitate was then dissolved in a 0.1 M $NaHCO_3$, followed by the biosurfactant extraction step with a solvent having a 2:1 CH_3Cl-to-C_2H_5OH ratio at room temperature (Zhang and Miller, 1992). The organic phase was transferred to a round bottom flask connected to a rotary evaporator to remove the solvent, yielding a viscous honey-colored biosurfactant product. About 5.20 g of the biosurfactant was extracted per liter of culture medium.

3.3.4 Fractionation of Biosurfactant

The key components of the biosurfactant were fractionated using a HPLC instrument (an Alltech 580 autosampler, an Alltech HPLC pump, model 626, and an Inertsil® ODS-3 column) equipped with an ELSD (Alltech, 2000ES). The mobile phase solutions were an aqueous solution of 10% acetonitrile (A) and pure acetonitrile (B). Both eluents contained 0.1% trifluoroacetic acid. The gradient system was used, starting with B from 30% to 70% in 5 min and then from 70% to 90% in 15 min. After that, the gradient of B was raised again to 100% at the end of the process. The flow rate of the mobile phase was set constant at 0.5 ml/min and the sample injection volume was 50 µl. The ELSD drift tube temperature was maintained at 100°C while the nebulizer flow rate was kept constant at 1.5 l/min (Noordman *et al.*, 2000). The gradual change in the affinity of the mobile phase resulted in the fractionation of the components in the biosurfactant. All fractions eluted from the HPLC column at different retention times were collected. Each fraction was then evaporated to remove all of the eluents to obtain a high-purity biosurfactant-containing material.

3.3.5 <u>Structural Characterization of Biosurfactant</u>

The chemical structures of the components in the biosurfactant sample were determined using FT-IR spectroscopy, NMR analysis, and mass spectrometry.

3.3.5.1 Fourier Transform Infrared Spectroscopy

FT-IR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures (Rodrigues, *et al.*, 2006). In this present study, a Thermo Nicolet Nexus 670 FT-IR spectrometer equipped with an attenuated total reflectance (ATR) crystal accessory was used to provide a chemical analysis of the components in the biosurfactant. The spectra were collected at a resolution of 4 cm^{-1} and 32 scans with correction for atmospheric carbon dioxide.

3.3.5.2 Nuclear Magnetic Resonance Analysis

The ^{1}H NMR spectra of each fraction from the fractionation step were achieved from an FT-NMR 500 mHz spectrometer (JEOL, JNM-A500) using deuterated chloroform as a solvent for all samples.

3.3.5.3 Mass Spectrometry

The isolated fractions eluted from the HPLC column were directly subjected to analysis with mass spectrometry. The experiments were performed with a Waters mass spectrometer using electrospray in the positive mode and a scanning mass range of 300–900 Da.

3.3.6 <u>Physicochemical Characterization of Biosurfactant</u>

The physicochemical properties of the biosurfactant product and its isolated fractions were investigated in comparisons with those of two commercial surfactants, Pluronic F-68 and SDS. Pluronic F-68 is the commercial trade name of a triblock nonionic surfactant composed of ethylene oxide (PEO) and propylene oxide (PPO) blocks in the form of PEO-PPO-PEO, while SDS is a widely used anionic surfactant.

3.3.6.1 Oil Displacement Test

The oil displacement test is a method used to measure the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on an oil-water interface. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant. The oil displacement test was done by adding 50 ml of distilled water to a petri dish with a diameter of 15 cm. After that, 20 μl of crude oil was dropped onto the surface of the water, followed by the addition of 10 μl of an aqueous solution containing a surfactant concentration of 20 mg/ml, onto the surface of the oil. The diameters of the clear zones of triplicate experiments from the same surfactant sample were determined for an averaged value of the clear zone diameter (Rodrigues, *et al.*, 2006).

3.3.6.2 Surface Tension Measurement

The surface tension of the aqueous solution at different surfactant concentrations was measured by using a du Nöuy ring-type tensiometer (Krüss, K10T). The surface tension measurement was carried out at 25±1°C after dipping the platinum ring in the solution for a while to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. The critical micelle concentration (CMC) was then determined from the break point of the surface tension versus its log of bulk concentration curve. For the calibration of the instrument, the surface tension of the pure water was measured before each set of experiments.

3.3.6.3 Measurement of Emulsification Activity

A mixture of 6 ml of the studied hydrocarbon or oil and 4 ml of 1 mg/ml of the biosurfactant or Pluronic F-68 or SDS was vortexed at a high speed for 2 min. The emulsion activity was investigated after 24 h and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg, 1987). The higher the emulsification index, the higher the emulsification activity of a tested surfactant.

3.3.6.4 Stability Testing

The biosurfactant sample and the two synthetic surfactants were prepared at the CMCs for the thermal stability test. The prepared surfactant solutions were incubated in a water bath at different temperatures and different time intervals before cooling to room temperature. The pH stability was investigated by adjusting the solutions to different pH values. For both thermal and pH stability testing, the surface tension was measured and used to indicate the stability.

3.3.7 Solution Properties and Vesicle Formation of Biosurfactant

3.3.7.1 Preparation of Biosurfactant Solution

The aqueous solution of the extracted biosurfactant was prepared by using a direct dissolution method. A specific amount of the extracted biosurfactant was simply dissolved in the PBS solution (pH 7.4) either in the absence or presence of the additive (NaCl or C_2H_5OH) to any desired concentration. To achieve a homogeneous solution, the sample was vortexed at room temperature, and was then filtered through a 0.45-μm pore size nylon filter at least two times in order to remove any dust. All of the measurements were performed one day after sample preparation, and no measurement was done on a prepared solution which exhibited two separate macroscopic phases.

3.3.7.2 Surface Tension Measurement

The surface tension of the PBS solution either in the absence or presence of the additives at different biosurfactant concentrations were measured by a drop shape analysis system (Krüss, DSA10 Mk2). The surface tension measurements were carried out at room temperature (25–27°C) by using the pendant drop method. All of the measurements were repeated three times and their average values were used. The critical micelle concentration (CMC) was then determined from the break point of surface tension *versus* its log of bulk concentration curve. For the calibration of the instrument, the surface tension of pure water was measured before each set of experiments.

3.3.7.3 Turbidity Measurement

The turbidity of the prepared biosurfactant solutions was measured by using a UV/Vis spectrophotometer (Shimadzu, UV-2550) at room temperature. The reported values were corresponded to the absorbance at a wavelength (λ) equal to 600 nm (Sánchez *et al.*, 2007).

3.3.7.4 Electrical Conductivity Measurement

It is known that the change in the electrical conductivity of a surfactant-containing solution relates to the aggregation of the surfactant molecules (Zana and Michels, 1998; Fan *et al.*, 2006); therefore, the electrical conductivity of the biosurfactant solution was determined using a dynamic light scattering instrument (Brookhaven, ZetaPALS) at 25±1°C.

3.3.7.5 Dynamic Light Scattering Measurement

The DLS technique was employed to measure the sizes of the biosurfactant vesicles formed at various conditions. The same dynamic light scattering instrument (Brookhaven, ZetaPALS) was used at 90° scattering angle and 25±1°C. The sizes of the biosurfactant vesicles were calculated from the diffusion coefficients obtained from computer analysis using the software provided with the instrument.

3.3.7.6 Transmission Electron Microscopy Examination

The morphology of the biosurfactant vesicles was observed with TEM by using the negative-staining technique. A drop of the test biosurfactant solution sample was placed onto a copper grid, and was stained with 1% uranyl acetate aqueous solution. The excess of the biosurfactant solution was removed by adsorbing the drop with a piece of filter paper. The grid was dried in a vacuum desiccator for at least 6 h. The samples were imaged under a transmission electron microscope (JEOL, JEM-2100).

3.3.7.7 Encapsulation Experiment

The encapsulation efficiency of the rhamnolipid vesicles was evaluated by using Sudan III, a water-insoluble dye, as a model lipophilic compound. A stock solution of Sudan III was prepared in chloroform at a desired concentration. Then, 100 μl of the stock solution was added to an empty vial and the solvent was evaporated

in a vacuum oven until dry. After that, 10 ml of the biosurfactant solution was added and allowed to equilibrate for one week. The residual insoluble dye was separated from the mixture by centrifugation at 10,000 rpm for 10 min. The colored supernatant was further filtered through a 0.45-μm pore size nylon filter at least two times, and the amount of the entrapped dye was then determined from the absorbance at a wavelength of 512 nm by using the UV/Vis spectrophotometer. The concentration of the entrapped dye was calculated by using a calibration curve of Sudan III prepared in chloroform in the concentration range of 0.10 to 12.30 mg/l. The encapsulation efficiency (*E*%) was defined as the ratio of the concentration of the entrapped Sudan III to the initial dye concentration.

3.3.8 Preparation of Rhamnolipid Vesicles as Potential Nanocarrier Systems

3.3.8.1 Preparation of Rhamnolipid Vesicles

The rhamnolipid vesicles were prepared in a PBS solution (pH 7.4) at a biosurfactant concentration of 0.13 wt.% (6.5 times the CMC) with various cholesterol concentrations using the thin film hydration method. Briefly, the extracted biosurfactant and cholesterol at any desired concentrations were dissolved in chloroform. The solvent was evaporated in a vacuum oven until dry, and the obtained film was subsequently hydrated with the PBS solution to achieve a constant biosurfactant concentration of 0.13 wt.%. The sample was vortexed at room temperature to get a homogeneous solution before being filtered through a 0.45-μm pore size nylon filter at least two times to remove any solid particles. The sample was left at room temperature for a day to ensure equilibration before taken for measurements and analysis of vesicle formation.

3.3.8.2 Turbidity Measurement

The turbidity of the biosurfactant solutions at various cholesterol concentrations was measured by using a UV/Vis spectrophotometer (Shimadzu, UV-2550) at room temperature. The reported values were corresponded to the absorbance at a wavelength (λ) of 600 nm (Sánchez *et al.* 2007).

3.3.8.3 Zeta Potential Measurement

The zeta potential of the vesicles formed in the biosurfactant solutions prepared at different cholesterol concentrations was measured by using a dynamic light scattering instrument (Brookhaven, ZetaPALS) at 25±1°C.

3.3.8.4 Dynamic Light Scattering Measurement

The dynamic light scattering (DLS) technique was used to measure the size of the rhamnolipid vesicles at various cholesterol concentrations. The DLS instrument (Brookhaven, ZetaPALS) was operated at 90° scattering angle and 25±1°C. The size of the rhamnolipid vesicles was expressed in terms of hydrodynamic diameter, which was calculated from the diffusion coefficients obtained from computer analysis by using the software provided with the instrument.

3.3.8.5 Transmission Electron Microscopy Examination

The morphology of the rhamnolipid vesicles was observed with transmission electron microscopy (TEM) using the negative-staining technique. A drop of the test biosurfactant solution sample was placed onto a copper grid and was stained with a 1% uranyl acetate aqueous solution. The excess sample solution was adsorbed with a piece of filter paper. The grid was dried in a vacuum desiccator for at least 6 h. The samples were imaged under a transmission electron microscope (JEOL, JEM-2100). The diameter of the rhamnolipid vesicles was then determined from TEM micrographs using SemAfore software, version 5.00 (JEOL (Skandinaviska) AB, Sweden).

3.3.8.6 Encapsulation Experiment

The *E*% of the rhamnolipid vesicles was evaluated by using Sudan III, a water-insoluble dye, as a model lipophilic compound. A stock solution of Sudan III was prepared in chloroform at a desired concentration. Then, 100 μl of the stock solution was added to an empty vial and the solvent was evaporated in a vacuum oven until dry. After that, 10 ml of the biosurfactant solution was added and allowed to equilibrate for one week. The residual insoluble dye was separated from the mixture by centrifugation at 10,000 rpm for 10 min. The colored supernatant was further filtered through a 0.45-μm pore size nylon filter at least two times, and the amount of the entrapped dye was

then determined from the absorbance at a wavelength of 512 nm by using the UV/Vis spectrophotometer (Shimadzu, UV-2550). The concentration of the entrapped dye was calculated by using a calibration curve of Sudan III prepared in chloroform in the concentration range of 0.3 to 35 μM. The *E*% was defined as the ratio of the concentration of the entrapped Sudan III to the initial dye concentration.

3.3.9 Biosurfactant Adsorption onto Polymeric Materials

3.3.9.1 Preparation of Polymeric Substrates

The diluted silk fibroin solution was used to prepare the silk fibroin film by either the spin coating or the solvent casting technique. After the solvent was evaporated at room temperature, the obtained film was treated with a 90% (v/v) CH_3OH solution for 10 min to induce the formation of the β-sheet structure, an insoluble form of the silk fibroin. The CH_3OH-treated silk fibroin film was excessively washed with distilled water and was left to dry at room temperature.

To prepare the chitosan film, a specific amount of chitosan platelets was dissolved in a 1% (v/v) CH_3COOH solution to the desired concentration. The chitosan solution was used to prepare the chitosan film by either the spin coating or the solvent casting technique. After the solvent was evaporated at room temperature, the obtained film was neutralized with a 1 M NaOH solution. The neutralized chitosan film was washed with distilled water and was left to dry at room temperature.

3.3.9.2 Surface Plasmon Resonance Analysis

The adsorption of the rhamnolipid biosurfactant onto the polymeric surfaces was studied using the SPR analysis—a well-known powerful method for measurement of thin films on the basis of an excitation of the surface plasmons by p-polarized light at the noble metal–dielectric interface (Miller *et al.*, 1992; Wagner *et al.*, 1995; Levchenko *et al.*, 2002). Surface plasmons, which are electromagnetic evanescent waves, propagate along the surface with a penetration depth range of 150–200 nm (Brandani and Stroeve, 2003), and the obtained SPR signal, expressed as the angle shift, is proportional to the amount of the adsorbed substance on the surface (Levchenko *et al.*,

2002). In the present work, thin films of either silk fibroin or chitosan was spun cast at the polymer concentration of 0.06% (w/v) at a speed of 1000 rpm on a glass slide coated with a 50-nm gold film. The SPR instrument (AUTOLAB, ESPRIT) was operated at room temperature. The p-polarized light was generated from a monochromatic laser light source at a wavelength of 670 nm. The flow rate across the surface during the adsorption period was 16.7 μl/s while the draining and the washing speeds were set constant at 250 μl/s. The sample injection volume was 50 μl. The SPR angle shift was reported after washing the surface with the fresh PBS solution to remove any excess biosurfactant molecules. The amount of the biosurfactant adsorbed onto the substratum surfaces was roughly estimated from the SPR angle shift using the following relationship: a change in the SPR angle shift of 120 millidegrees (mDA) represents the adsorbed mass of 1 ng/mm^2 (Kausaite *et al.*, 2007). Dividing the adsorbed mass with an average molecular weight of the biosurfactant, the amount of the biosurfactant adsorbed onto the substratum surfaces was expressed as $\mu mol/m^2$.

3.3.9.3 Quartz Crystal Microbalance with Dissipation Monitoring

The QCM-D experiment was carried out by using a Q-sense E1 system. Prior to the measurement, the single sensor crystal with a gold coating and the rubber O-ring sealed between the sensor and the cell were cleaned by the ultrasonication in a 2% (v/v) anionic/nonionic surfactant (Decon 90) solution for 15 min. After a thorough rinse with distilled water, both the sensor and the O-ring were ultrasonicated in distilled water for 15 min before drying under a nitrogen flow. Then only the sensor was further cleaned in a UV/ozone chamber (BioForce Nanosciences, UV/Ozone ProCleaner^{TM}) for 20 min. After the cleaning process, both the sensor and the O-ring were assembled in the QCM-D instrument. The polymer solution (either silk fibroin or chitosan) at the concentration of 0.06% (w/v) was first injected into the QCM-D instrument to form a self-assembly polymer layer on the sensor surface. The weakly bound polymer molecules were removed from the sensor surface by thoroughly rinsing with a proper solvent—the distilled water (for silk fibroin) or the 1% (v/v) CH_3COOH solution (for chitosan). For the silk fibroin, the methanol treatment was also performed

to induce the formation of the β-sheet structure. The biosurfactant solutions were injected at various concentrations. To remove any excess biosurfactant aggregates on the polymer layer, the fresh PBS solution was subsequently injected into the QCM-D instrument after the adsorption period. The flow rate of the test sample was set constant at 0.1 ml/min, and the temperature was controlled at 25°C. After excluding the amount of the pre-adsorbed polymer layer, the amount of the adsorbed biosurfactant was calculated from a change in the third overtone of the resonance frequency by applying the Sauerbrey equation using the software provided with the instrument. However, the mass obtained from the QCM-D technique also includes the amount of the solvent bound to the adsorbed layer; therefore, the adsorbed mass determined from the QCM-D is always much higher than that measured by other analytical techniques, including the SPR analysis (Sakai *et al.*, 2006). In the present work, the adsorbed masses obtained from both the QCM-D experiment and the SPR analysis were combined to get the quantitative information about the degree of hydration of the adsorbed layer on the studied substratum surfaces using the following equation:

$$\text{Water Content (\%)} = \frac{(\Gamma_{QCM-D} - \Gamma_{SPR})}{\Gamma_{QCM-D}} \times 100$$

where $\Gamma_{QCM\text{-}D}$ and Γ_{SPR} are the adsorbed masses obtained from the QCM-D experiment and the SPR analysis, respectively.

3.3.9.4 Surface Modification of Polymeric Substrates

The polymeric substrate (either silk fibroin or chitosan film) was immersed in the biosurfactant solution at the desired concentration at room temperature for 24 h. After that, the polymeric substrate was repeatedly rinsed with the PBS solution to remove any excess biosurfactant molecules on the substratum surfaces. The surface-modified substrate was dried under a nitrogen flow before being analyses.

3.3.9.5 Water Contact Angle Measurement

The contact angle formed between the 30-μl water drop and the polymeric surface was measured by a drop shape analysis system (Krüss, DSA10 Mk2). The contact angle measurements were carried out at room temperature using the sessile

drop technique. The reported values were averages of at least three measurements made at different places of the test surfaces.

3.3.9.6 Surface Probe Microscopy

The surface topography of the polymeric substrates was investigated by using a scanning probe microscope (SPM) (Veeco, Nanoscope IV) with the tapping mode in air at room temperature. The obtained micrographs were minimally flattened, and high frequency noise was diminished in order to facilitate data analysis.

3.3.9.7 Cytotoxicity Tests

The cytotoxicity tests of both unmodified and surface-modified polymeric films against human dermal fibroblasts and keratinocytes were carried out at bioassay laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. Both types of cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) of fetal bovine serum, 2 mM of L-glutamine, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin. The cell cultures were incubated at 37°C in a fully humidified air atmosphere with 5% of CO_2. When the cells reached 80% confluence, they were serially sub-cultured and only the cells from the fifth passage were used.

Either unmodified or surface-modified polymeric substrate was first prepared in a well of the 24-well plate. The cells were seeded at a density of 10,000 cells/well, and were incubated at 37°C and 5% of CO_2 for the desired time interval. For the fibroblasts, the cytotoxicity was examined using the resazurin assay, the fluorescent-dye based technique (Brien *et al.*, 2000). After the cells were cultured for 24 h, 100 μl of the resazurin dye solution at the concentration of 62.5 μg/ml was added to each culture well. The fluorescent signal was measured everyday for 3 days at the excitation stage of 530 nm and the emission stage of 590 nm using the multi-detection microplate reader (Molecular Devices, SpectraMax M5). For the keratinocytes, the experiment was performed by using the methyltetrazolium (MTT) assay; a tetrazolium-dye based colorimetric microtitration (Plumb *et al.*, 1989). The cytotoxicity was examined after the

cells were cultured for 1 day and 7 days. Briefly, the MTT solution at the concentration of 5 mg/ml was added into each well before being further incubated for 4 h. The culture medium containing the MTT solution was then removed, and the formazan crystals were solubilized in a dimethylsulfoxide (DMSO) solution. The optical density of the formazan solution was measured at the wavelength of 570 nm using the multi-detection microplate reader (Molecular Devices, SpectraMax M5). The cells cultured in the absence of polymeric substrates were served as the control. The morphology of the cells attached on the substratum surfaces was also observed under an optical light microscope.

3.3.10 Statistical Analysis

The experimental data are presented in terms of arithmetic averages of at least three replicates and the standard deviations are indicated by the error bars. The analyses were done using SigmaPlot software, version 8.02 (SPSS Inc., UK).