CHAPTER III

EXPERIMENTAL

3.1 Materials

1. Acetic acid	: Carlo
2. Acrylic acid (AA)	: Sigma-Aldrich
3. Chitosan (MW=15,000, 83,000)	: Seafresh Chitosan (Lab) Co.,Ltd.
4. Collagen marine	: Ta Ming Enterprises Co.,Ltd.
5. Collagen type I from calf skin acid	: Sigma
6. Collagen type IV from human placenta	: Sigma
7. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI)	: Fluka
8. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)	: Sigma
9. Dimethylsulfoxide (DMSO)	: Merck
10. *N, N'*-disuccinimidyl carbonate (DSC)	: Novabiochem
11. Dulbecco's phosphate-buffered saline (D-PBS) (no calcium or magnesium)	: Gibco
12. 1,4-Dioxane	: Carlo
13. Epidermal growth factor	: Gibco
14. Ethanol	: Merck
15. Fibroblast (L929) cell line	: ATCC
16. Fetal bovine serum (FBS)	: InVitromex
17. Gentamycin	: M&H manufacturing Co.,Ltd.
18. Glycine	: Sigma
19. Human epidermal keratinocyte (HEK001) cell line	: ATCC

20. Hydrogen peroxide : Merck
21. 1,6-hexamethylenediamine : Fluka
22. *N*-hydroxysuccinimide (NHS) : Fluka
23. Isopropanol (IPA) : Merck
24. Keratinocyte-serum free medium : Gibco
25. L-glutamine : Gibco
26. Ninhydrin : Fluka
27. Normal saline : General Hospital Products Public Co., Ltd.
28. Penicillin G Sodium : General Drugs House Co.,Ltd.
29. Phosphate buffer saline (PBS) : Sigma
30. Polycaprolactone (PCL, M_w = 65,000) : Aldrich
31. RPMI 1640 medium : InVitromex
32. Sodium hydroxide : Merck
33. Streptomycin sulfate : M&H manufacturing Co.,Ltd.
34. Toluidine Blue O : Aldrich
35. Triethanolamine : Aldrich
36. Trypsin-EDTA solution : Sigma

3.2 Equipments

3.2.1 Contact Angle Measurements

Contact angle goniometer model 100-00 and a Gilmont syringe with a 24-gauge flat-tipped needle (Ramé-Hart, Inc., USA) was used for the determination of water contact angles. The measurements were carried out in air at the room temperature. Dynamic advancing and receding angles were recorded while water was added to and withdrawn from the drop, respectively. The reported angle is an average of 5 measurements on different area of each sample.

3.2.2 Photochemical Reactor

The Rayonet Photochemical Chamber Reactor model RPR-100 equipped with the Rayonet Merry-Go-Round model-RMA-400 was used to generate UV-A light with a wavelength at 350 nm for photo-oxidation and graft copolymerization step.

3.2.3 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.

3.2.4 UV-Spectrometer

UV spectroscopy Model Techna, specgene was used for determination of the amount of amino group using ninhydrin method and carboxyl group using toluidine blue O method on the modified PCL surface by reading the absorbance at 538 and 633 nm, respectively.

3.2.5 Microplate Reader

Microplate Reader Model MK II, Titertek Multiskan MCC/340, Finland was used for determination the optical density of samples in MTT assay.

3.2.6 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.

3.2.7 Statistical Analysis

Values are expressed as the mean ± SD. Experiments were 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 12 software. $p<0.05$ was considered statistically significant.

3.3 Methods

3.3.1 Preparation of Polycaprolactone Films

Polycaprolactone (PCL) film was prepared by dissolving PCL 1.5 g in 25 mL 1,4-dioxane and cast into a glass Petri dish (diameter 9.6 cm). The solvent was evaporated at room temperature overnight, and further dried under vacuum for another 24 h at 30°C. A translucent PCL film with a thickness of about 200 μm was obtained.

3.3.2 Surface Modification of PCL Film *via* Aminolysis

The film was cut into pieces of 1x4 cm^2 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 1,6-hexamethylene diamine/isopropanol (IPA) solution having a predetermined concentration for a given time at 37°C. The resulting aminolyzed PCL film 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.

3.3.3 Determination of the Amino Groups on Aminolyzed PCL Surface

The ninhydrin analysis method was employed to quantitatively detect the amount of NH_2 groups on the aminolyzed PCL film. The film (1×1 cm^2) was immersed in 1 M ninhydrin/ethanol solution for 1 min and placed into a glass tube, followed by heating at 80°C for 15 min to accelerate the reaction between ninhydrin and amino groups on PCL film. After the adsorbed ethanol had evaporated, 1 mL of 1,4-dioxane was added into the tube to dissolve the film when the film surface turned blue. A 1 mL of isopropanol was then 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-hexamethylene diamine in 1,4-dioxane/isopropanol (1:1, v/v) solution.

3.3.4 Activation of Aminolyzed PCL and Immobilization of Collagen or Chitosan

Aminolyzed PCL film (0.9×1 cm^2) was immersed in 0.1 M *N,N'*-disuccinimidyl carbonate (DSC)/dimethylsulfoxide (DMSO) solution in the presence of 0.1 M triethylamine for a given time at ambient temperature followed by rinsing with large amount of deionized water. The film was then directly transferred to 1 mL collagen/phosphate buffer saline (PBS) solution or chitosan/acetic acid solution at ambient temperature for 24 h. Aminolyzed PCL-biomolecule film was rinsed by soaking in deionized water for 24 h (for aminolyzed PCL-collagen) or by 1% acetic acid followed by soaking in deionized water for 24 h (for aminolyzed PCL-chitosan) and dried under vacuum before surface characterization.

3.3.5 Surface Modification of PCL Film via Photo-oxidation and Graft Copolymerization

The film was cut into pieces of 1×4 cm^2 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 a quartz tube containing 6 mL hydrogen peroxide solution (30%). Under rotation by a motor, the tube was

exposed to UV light generated from high-pressure mercury lamp (λ=350 nm) for a given time at 30°C. The photo-oxidized film was rinsed with deionized water thoroughly to eliminate the free hydrogen peroxide, and then placed in a copolymerization quartz tube containing 6 mL acrylic acid (AA) aqueous solution having a predetermined concentration. The solution in the tube was degassed by a continuous flow of nitrogen throughout the reaction. Graft copolymerization was carried out under UV irradiation from the mercury lamp for a given time at 30°C. The grafted film (PCL-*g*-PAA) was rinsed with a large amount of deionized water for at least 24 h at 37°C to remove the adsorbed homopolymer, and subsequently dried under vacuum at 30°C to constant weight.

3.3.6 Determination of the Carboxyl Groups on PCL-*g*-PAA Surface

The toluidine blue o staining method was employed to determine the amount of COOH groups on PCL-*g*-PAA. A 0.5 mM dye solution was prepared at pH 10, and the grafted film was placed in this solution for 6 h at 30°C. The film was then removed and thoroughly washed with a sodium hydroxide solution of pH 9 for 24 h to remove any noncomplexed dye adhering to the surface. The dye was desorbed from the film in a 50% acetic acid solution for 16 h, and the final dye content was obtained by the measurement of the optical density of the solution at 633 nm with an UV-vis spectrophotometer. The PAA content (grafting density) was obtained from a calibration plot of the optical density versus dye concentration assuming that one dye molecule reacts stoichiometrically with one carboxyl group.

3.3.7 Activation of PCL-*g*-PAA and Immobilization of Collagen or Chitosan

PCL-*g*-PAA film (0.9×1 cm^2) was treated with the mixture of *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI). The EDCI solution was prepared by dissolving 0.0767 g of EDCI in 1 mL of MilliQ water. The NHS solution was prepared by dissolving 0.0115 g of NHS in 1 mL of MilliQ water. Mixing the two solutions gave a mixture

containing 0.4 M EDCI and 0.1 M NHS. The PCL-*g*-PAA film was immersed in the mixture and allowed to react for a given time at ambient temperature, followed by rinsing with large amount of deionized water. The sample was then directly transferred to 1 mL of collagen/phosphate buffer saline (PBS) solutions or chitosan/acetic acid solution having a predetermined concentration for a given time at the same temperature to achieve biomolecule immobilization. PCL-*g*-PAA-biomolecule film was rinsed by soaking in deionized water for 24 h (for PCL-*g*-PAA-collagen) or by 1% acetic acid followed by soaking in deionized water for 24 h (for PCL-*g*-PAA-chitosan) and dried under vacuum before surface characterization.

3.3.8 Determination of the Amino Groups on PCL surface after Collagen or Chitosan Immobilization

The ninhydrin analysis method was employed to quantitatively detect the amount of NH_2 groups on the biomolecule-immobilized PCL films. The film (0.9×1 cm) was immersed in 1 M ninhydrin/ethanol solution for 1 min and then was placed into a glass tube, followed by heating at 80°C for 15 min to accelerate the reaction between ninhydrin and amino groups on biomolecule-immobilized PCL film. After the adsorbed ethanol had evaporated, 0.5 mL of 1,4-dioxane was added into the tube to dissolve the film when the film surface turned blue. A 0.5 mL of isopropanol 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/isopropanol (1:1, v/v) solution.

3.3.9 Cell Study

Fibroblast (L929) and human epidermal keratinocyte (HEK001) cell lines were used with the seeding density of 5,000 and 10,000 cells/cm^3, respectively. The L929 cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), penicillin (100,000 U/L) and streptomycin (100 mg/L) while the HEK001 were cultured in keratinocyte-serum free medium supplemented with 5

ng/mL human recombinant EGF, 2 mM L-glutamine (without bovine pituitary extract and without serum), penicillin (100,000 U/L) and streptomycin (100 mg/L).

Both modified and unmodified PCL films (diameter 6 mm) were sterilized by soaking in 70% ethanol in water for 30 min and washed twice with a medium containing gentamycin (200 mg/L), penicillin (200,000 U/L) and streptomycin (200 mg/L). The films were then transferred to cover the bottom of 96-well tissue culture polystyrene plate. The 3 replicated samples were used for each condition. Approximately 5×10^3 L929 cells or 1×10^4 HEK001 cells in 0.2 mL culture medium was pipetted into each well containing films as well as into the bottom of tissue culture polystyrene (TCPS) plates as a control and then incubated under 5% CO_2 at 37°C.

MTT assay was used to investigate cell adhesion and proliferation. After 12 h of incubation, the culture medium was removed to discard the unattached cell and the 0.2 mL fresh culture medium was pipetted into each well followed by 10 µL of 5 mg/mL MTT/normal saline solution. After incubation for 4 h, the supernatant solution was removed and 150 µL of DMSO was pipetted into each well to dissolve the purple crystals of formazan. Next, 25 µL of 0.1 M glycine (pH 10.5) was added. The optical density of sample was measured using microplate reader at a wavelength of 540 nm. Cell adhesion ratio on each surface was evaluated using the equation shown below where $OD_{\text{sample, 12 h}}$ represents the optical density on the different polymeric surfaces and $OD_{\text{TCPS, 12 h}}$ represents the optical density on TCPS surfaces, which was utilized as a standard.

$$\text{Cell adhesion ratio (\%)} = \frac{OD_{sample,12h}}{OD_{TCPS,12h}} \times 100 \quad \text{.....(3.1)}$$

The measurement of change in number of proliferated cells on modified and unmodified PCL films was determined at the incubation time point of 48 and 96 hours. The cell proliferation ratio on each sample was evaluated by using the following equation.

$$\text{Cell proliferation ratio (\%)} = \frac{OD_{sample,48or96h}}{OD_{TCPS,48or96h}} \times 100 \quad \text{.....(3.2)}$$