CHAPTER III

EXPERIMENTAL

3.1 Materials

All reagents and materials are analytical grade and use without further purification

1. Acetic acid : Merck
2. Bromine : Fluka
3. Chloroform-d : Aldrich
4. Deuterium oxide : Aldrich
5. Dicyclohexyl carbodiimide (DCC) : Fluka
6. Diethylether : Merck
7. 1,4-Dioxane : Merck
8. Ethanol : Merck
9. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide : Fluka
10. Fibroblast cell line (B95) : Allergy and Clinical Immunology Unit, Faculty of Medicine Chulalongkorn University.
11. H-Arg-Gly-Asp-OH : CalBiochem
12. H-Arg-Gly-Asp-Ser-OH : CalBiochem
13. H-Gly-Arg-Gly-Asp-Ser-OH : CalBiochem
14. Heptafluorobutyryl chloride : Aldrich
15. *N*-hydroxysuccinamide (NHS) : Fluka
16. Isopropyl alcohol : Merck
17. L-tyrosine : Fluka
18. Methanol : Merck
19. Methylene chloride : Merck

20. Ninhydrin : Merck
21. Phosphate buffer saline (PBS) : Aldrich
22. Poly(DTE-*co*-20%DT carbonate) Mw 81126 : The New Jersey Center For Biometerials
23. RPMI 1640 : Gibco
24. Sodium metabisulphite : Unival
25. Tetrahydrofuran : Merck
26. Triethylamine : Merck
27. Trypsin-EDTA solution : Gibco

3.2 Equipments

3.2.1 Nuclear Magnetic Resonance (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded in either $CDCl_3$, D_2O or DCl using a Bruker, model AC-F200, Avance DPX-400 and Varian, model Mercury-400 nuclear magnetic resonance spectrometer operating at 400 MHz. Chemical shifts (δ) are reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.2 Contact Angle Measurements

Contact angle meter model FACE, Japan was used for the determination of water contact angles. A droplet of testing Milli-Q water is placed on the tested surface by bringing the surface into contact with a droplet suspended from a needle of the syringe. 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 Dynamic advancing and receding angles were recorded while water was added to and withdrawn from the drop, respectively.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.

3.2.3 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectra were collected using ESCA-200, SCIENTA, Uppsala, Sweden. In this study, the take off angle at 15° and 90° were chosen and the approximate of depth profile is ~10 Å and ~40 Å, respectively.

3.2.4 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

All spectra were collected at resolution of 4 cm^{-1} and 64 scan using Bruker vector 33 FT-IR spectrometer equipped with a DTGS detector. A single attenuated total reflection accessory with 45° zinc selenide (ZnSe) IRE (spectra Tech, USA) and a varible angle reflection accessory (Seagull^{TM}, Harrick Scientific, USA) with a hemispherial ZnSe IRE were employed for all ATR spectral acquisitions.

3.2.5 UV-Spectroscopy

UV spectroscopy Model Techna, specgene was used for determination the amount of amino groups on poly(DTE-*co*-20%DT carbonate) surface after RGD immobilization using ninhydrin method by reading UV absorbance at 538 nm.

3.2.6 Hemocytometer

The Hemocytometer is a cheap and effective way to count cells. This apparatus is basically a microscope slide consisting of an optically flat chamber. A coverslip is then place securely over the chamber thereby determining its depth. A known volume (20μL) of a homogenous cell suspension is then drawn into the chamber by capillary action. The microscope is used to focus on a defined grid area of dimension 1mm^2 and the numbers of cell in the area counted.

In order to achieve an accurate cell count it is essential that the suspended cells are homogenous and devoid of aggregation. It is also important to transfer the cells from gilsen tip to chamber as quickly as possible to avoid the settling or adhering of cells to tip.

Ideally the numbers of cells should be between 100-300/mm^2 in order to provide a reasonable degree of accuracy. The greater the number of cells the better. Counts of both chambers should be taken and average calculated.

The number of cells is calculated using the formula below:-

$$c = n/v$$

Where c – cell concentration(cells/mL)

n – number of cells counted

v – volumn counted(mL)

The Improved Neubauer Slide which has a depth of chamber of 0.1mm is used. Therefore using the count area of $1mm^3$ (or $10^{-4}cm^3$). Rearranging the above formula in light of this information gives:-

$$C = n \times 10^4$$

Therefore if there is an average cell count (n) of 200 then the cell concentration will be 200×10^4 which is equivalent to 2,000,000 cells/mL. The total numbers of cells present can be simply calculated by multiplying c by the original volume of suspended cells.

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 Student-Newman-Keul's test (SNK) multiple comparisons posttest using SPSS 10.0.1 software. $p<0.01$ or $p<0.05$ was considered statistically significant.

3.3 Experimental

3.3.1 Activation of Poly(DTE-*co*-20%DT carbonate) with *N*-hydroxysuccinimide under Homogeneous Condition

Poly(DTE-*co*-20%DT carbonate) 0.44 g in 10 mL of 10% methanol in methylene chloride (10 mL) was added into a 25 mL round bottom flask containing a magnetic bar. 0.029 g (0.25 mmole) of *N*-hydroxysuccinimide (NHS) and 0.052 g (0.25 mmole) dicyclohexyl-carbodiimide (DCC) were added. After reacting for 24 h at ambient temperature, precipitated dicyclohexyl urea was filtered and the solution was concentrated by evaporation. The white solids product was dried under vacuum.

3.3.2 Preparation of Poly(DTE-*co*-20%DT carbonate) Films

Poly(DTE-*co*-20%DT carbonate) sample (2.0 g) was dissolved in 20 mL of 10% methanol in dichloromethane. The solution was filtered by PTFE membrane (1.0 μm pore diameter) before cast into a glass mold (10x10 cm^2) coated with Teflon Solvent was allowed to evaporate under N_2 atmosphere overnight. The film was dried under vacuum for 2 days.

3.3.3 Activation of Poly(DTE-*co*-20%DT carbonate) with *N*-hydroxysuccinimide under Heterogeneous Condition

Poly(DTE-*co*-20%DT carbonate) film (5x5 mm^2) was treated with a carbodiimide solution (0.1M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) and 0.1 M *N*-hydroxysuccinimide (NHS)) in 5 mL selected solvent at ambient temperature for a certain period of time. After rinsed twice with ethanol, the activated poly(DTE-*co*-20%DT carbonate) film was dried under vacuum overnight.

3.3.4 Synthesis of L-3,5-Dibromotyrosine

0.9 g L-bromotyrosine was dissolved in 1 mL of glacial acetic acid and 9 mL of water. The solution was added dropwise to the solution of 0.64 mL of bromine in 3 mL of glacial acid in an ice bath. Sodium metabisulphite (NaS_2O_5) was then added to the solution until the color of bromine disappeared. After pH of solution was adjusted to 7, the product was isolated by filtration, washed thoroughly with cool deionized water and dried under vacuum.

H-NMR (DCl): δ 2.9 (2H, PhOHC$\underline{H}_2$, m, J = 5.86Hz, J = 7.62Hz) δ 4.03 (1H, PhOHCH$_2$C$\underline{H}$NH_2, t), δ 7.2 (2H, Ar$\underline{H}$CH_2, s)

3.3.5 Reaction of Activated Poly(DTE-*co*-20%DT carbonate) Film with L-3,5-Dibromotyrosine

L-3,5-dibromotyrosine was dissolved in a solution of 2 mL DMF and 3 mL phosphate buffer solution (pH = 7.4). The activated poly(DTE-*co*-20%DT carbonate) film was added to the solution. After reacting for 2 h, the film was rinsed twice with

phosphate buffer solution (pH = 7.4) and deionized water, respectively. The film was dried under vacuum overnight.

3.3.6 Reaction of Activated Poly(DTE-*co*-20%DT carbonate) Film with RGD-containing Peptide

RGD-containing peptide (RGD, RGDS or GRGDS; 0.05 M) was dissolved in phosphate buffer solution (pH = 7.4). The activated poly(DTE-*co*-20%DT carbonate) film was added to the solution. After reacting for 24 h, the film was rinsed twice with phosphate buffer solution (pH = 7.4) and deionized water, respectively. The film was dried under vacuum overnight.

3.3.7 Labeling of Immobilized RGD-containing Peptide by Heptafluorobutyryl chloride

Diethyl ether (10 mL) was added into a Schlenk flask containing an activated poly(DTE-*co*-20%DT carbonate) film. Triethylamine (0.070 mL, 0.05 mol) and heptafluorobutyryl chloride (0.075 mL, 0.05 mol) were added into the Schlenk flask via syringe. The solution was stirred at room temperature under nitrogen atmosphere for 24 h. Then the films were rinsed twice with diethyl ether and ethanol, respectively. The film was dried under vacuum overnight.

3.3.8 Determination of the Amino Groups on Poly(DTE-*co*-20%DT carbonate) Surface after RGD Immobilization

The ninhydrin analysis method was employed to quantitatively detect the amount of NH_2 groups on the RGD-immobilized poly(DTE-*co*-20%DT carbonate) film. The film was immersed in 1.0 mol/L ninhydrin/ethanol solution for 1 min and then was placed into a glass tube, following with heating at 80 °C for 15 min to accelerate the reaction between ninhydrin and amino groups on the 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 appeared blue. Another 0.5 mL of 2-propanol 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-hexanediamine in 1,4-dioxane/isopropanol (1:1, v:v) solution.

3.3.9 Cell Study

96-well cell culture plates were employed for cell study. Both modified and unmodified poly(DTE-*co*-20%DT carbonate) films (diameter: 6 mm) were transferred to cover the bottom of wells. 6 replicated samples were used for each condition. The seeding density of the fibroblast cell line (B95) was 25,000 cells/cm^3. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and incubated under 5% CO_2 at 37°C. As a control, the B95 were also directly seeded on tissue culture polystyrene substrate (TCPS) of plates. Medium was replaced 48 h after starting the culture and every 48 h thereafter.

After 12 h of incubation, the B95 were digested and isolated with 0.25% trypsin-EDTA for 10 min. Cells were counted with a hemacytometer, and the cell adhesion on each surface was evaluated using the equation shown below where $N_{\text{sample, 12 h}}$ represents the cell number on the different polymeric surfaces and $N_{\text{TCPS, 12 h}}$ represents the cell number on TCPS surfaces, which was utilized as a standard.

$$\% \text{ Cell adhesion ratio (CAR)} = \frac{N_{sample,12h}}{N_{TCPS,12h}} \times 100$$

The measurement of change in number of proliferated cells on modified and unmodified poly(DTE-*co*-20%DT carbonate) films was determined at the time point of 48 and 96 hours. Approximately 5 x 10^3 fibroblasts in 0.2 mL culture medium were pipetted into each well of 96-well tissue culture dishes containing the modified and unmodified poly(DTE-*co*-20%DT carbonate) films. Culture medium was replaced with fresh medium every 48 h while the cells were incubated, After designated incubation time, the films were rinsed with 0.2 mL phosphate buffer solution (pH = 7.4) to remove unattached cells and incubated with 0.25% trypsin-EDTA for 10 min to remove attached cells. Next, trypsin was neutralized with 0.2 mL phosphate buffer solution (pH = 7.4). Cells were counted with a hemocytometer.