CHAPTER III

EXPERIMENTAL

3.1 Materials

All reagents and materials are analytical grade and used without further purification.

1. Acetic acid : Merck
2. Acetone : Merck
3. Chitosan flakes, 95% DD ; MW = 100,000 : Seafresh Chitosan (Lab)
4. Dimethylsulfoxide (DMSO) : Merck
5. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) : Sigma
6. Ethanol : Merck
7. Fetal bovine serum (FBS) : InVitromex
8. Fibroblast (L929) cell line : ATCC
9. 5-Formyl-2-furansulfonic acid, sodium salt (FFSA) : Aldrich
10. Glycidyltrimethylammonium chloride (GTMAC) : Fluka
11. Glycine : Sigma
12. Hydrochloric acid : Fluka
13. Hydrogen peroxide : Merck
14. Methanol : Merck
15. 11-mercaptoundecanoic acid : Aldrich
16. 11-mercapto-1-undecanol : Aldrich
17. Milli-Q water : Milli-Q Lab system

18. Plasma-treated poly(ethylene terephthalate) film : Wako Pure Chemical Industry, Ltd.
19. Poly(acrylic acid), sodium salt (PAA), MW = 60,000: Fluka
20. Poly(allylamine hydrochloride) (PAH), MW = 70,000: Aldrich
21. Poly(sodium styrene sulfonate) (PSS), MW = 70,000: Aldrich
22. Sodium borohydride : Fluka
23. Sodium chloride : Merck
24. Sodium hydroxide : Carlo Erba
25. Streptomycin sulfate : M&H manufacturing
26. Succinic anhydride (SA) : May &Baker
27. Toluidine blue O (TBO) : Aldrich
28. Triethanolamine : Unilab
29. RPMI 1640 medium : InVitromex

3.2 Equipment

3.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^1H NMR spectra were recorded in CF_3COOH/D_2O or D_2O using Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts (δ) were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were recorded with a FT-IR spectrometer (Nicolet, USA), model Impact 410, with 32 scans at resolution 4 cm^{-1}. Data at frequencies of 400-4000 cm^{-1} were collected using a TGS detector.

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

All spectra were collected at a resolution of 4 cm^{-1} by 128 scans using a Nicolet Magna 750 FTIR 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 (Seagull™, Harrick Scientific, USA) with a hemispherical Ge IRE were employed for all ATR spectral acquisitions.

3.2.4 Quartz Crystal Microbalance (QCM)

The apparatus and the crystals used for QCM measurements were obtained from Maxtek, Inc. (USA). An AT-cut quartz crystal with a resonance frequency of 5 MHz, model SC-501-1 was used. The plating crystal (1 inch in diameter) was covered by evaporated gold on both faces. The frequency shift of the gold-coated QCM plate was monitored by a Maxtek plating monitor (model PM-710) coupled with an MPS-550 sensor probe. The Data-Log Software (Maxtek) was used for data acquisition and monitoring. The gold-coated QCM plate was cleaned by soaking in Piranha solution (3:1, H_2SO_4:30% H_2O_2) for 10 min, rinsing thoroughly with nanopure water and drying with a light stream of nitrogen gas prior to use. *Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution and only small quantities should be prepared.*

The surface modification of the gold-coated QCM plate was carried out by immersion in an ethanolic solution containing 1:1 (v/v) of 10 mM 11-mercaptoundecanoic acid and 10 mM 11-mercapto-1-undecanol at ambient temperature. After 24 h, the plate was rinsed with ethanol and dried with a light stream of nitrogen gas. For the measurements in the conventional mode, one side of the gold-coated QCM plate was covered with a designated polyelectrolyte solution for a desired period of time, rinsed with copious amounts of Milli-Q water, dried with a light stream of nitrogen gas and then the resonance frequency was measured in air. In the case of multilayer assembly, rinsing with copious amounts of Milli-Q water

and drying by a light stream of nitrogen gas were applied after each step of deposition.

3.2.5 Contact Angle Measurement

A contact angle goniometer model 100-00 equipped with a Gilmont syringe and a 24-gauge flat-tipped needle (Ramé-Hart, Inc., USA) was used for the determination of water contact angles. A droplet of Milli-Q water was placed on the tested surface by bringing the surface into contact with a droplet suspended from a needle on the syringe. The measurements were carried out in air at room temperature. The reported angle is an average of 5 measurements on different areas of each sample.

3.2.6 Atomic Force Microscopy (AFM)

AFM images were recorded with a Scanning Probe Microscope; model NanoScope®IV, Veeco, USA. Measurements were performed in air at ambient temperature using a tapping mode. Silicon nitride tips with a resonance frequency of 267-298 kHz and a spring constant 20-80 N/m were used.

3.2.7 Optical Density Analysis by Microplate Reader

Optical densities of the samples in MTT assay were recorded with Microplate Reader Model MK II, Titertek Multiskan MCC/340, Finland.

3.2.8 Statistical Analysis

Data are expressed as the means ± standard deviations (SD) of a representative of three similar experiments carried out in triplicate. Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 14.0 software. Statistical comparisons made by One-Way Analysis of Variance (AVOVA) with the Least Square Difference (LSD) tests were used for post

hoc evaluations of differences between groups. In all statistical evaluations, $p<0.05$ was considered as statistically significant.

3.3 Synthesis of Charged Derivatives of Chitosan

3.3.1 *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC)

HTACC was synthesized according to a method modified from that of Seong *et al.* [7]. Chitosan flakes (0.50 g, 1 equiv. of NH_2) were dissolved in 1.0 % (v/v) aqueous acetic acid (25 mL) to prepare a 2.0% (w/v) chitosan solution. GTMAC (0.90 g, 2 equiv.) was added. The reaction was performed at 70 °C for 24 h. After the reaction, the solution was poured into an acetone/ethanol (50:50, v/v) mixture to obtain the precipitate. The precipitate was filtered, washed thoroughly with acetone, dried under vacuum at room temperature and kept in a desiccator. The same procedure was used for the synthesis of HTACC using stoichiometric ratios between the NH_2 of chitosan and GTMAC of 1:4 and 1:6.

3.3.2 *N*-succinyl chitosan (SCC)

SCC was synthesized according to a method modified from that of Aoki *et al.* [23]. Chitosan flakes (0.50 g, 1 equiv. of NH_2) were dissolved in 1.0 % (v/v) aqueous acetic acid solution (25 mL) to prepare a 2.0 % (w/v) chitosan solution. The chitosan solution was diluted with 40 mL of methanol. SA (0.30 g, 1 equiv.) dissolved in a minimum amount of acetone, was then added to the chitosan solution. The mixture was vigorously stirred for 2 h at room temperature. The obtained viscous solution was diluted with 50 mL of water and the pH of the solution was adjusted to 10 using 2 M NaOH solution. After dialysis for 5 days, the solution was freeze-dried to obtain a cotton-like white material. The same procedure was used for the synthesis of SCC using stoichiometric ratios between the NH_2 of chitosan and SA of 1:2, 1:3, 1:4, 1:8, and 1:12.

3.3.3 *N*-sulfofurfuryl chitosan (SFC)

SFC was synthesized according to a method modified from that of Amiji [10]. Chitosan flakes (0.50 g, 1 equiv. of NH_2) were dissolved in 1.0 % (v/v) aqueous acetic acid (25 mL) to prepare a 2.0% (w/v) solution. Methanol (10 mL) containing 1.0% (w/v) triethanolamine was slowly added to the chitosan solution. The mixture was stirred for 6 h at room temperature. FFSA (0.30 g, 0.5 equiv.) was slowly added to the chitosan slurry. The reaction was allowed to proceed for 18 h at room temperature. As the reaction continued, the Schiff's base thus formed was reduced by a slow addition of sodium borohydride ($NaBH_4$) (0.70 g, 6 equiv.). After the reduction for 6 h, the Schiff's base slowly dissolved to form a viscous solution. SFC was precipitated in methanol and washed extensively with methanol and acetone to remove unreacted FFSA. The polymer was dried at room temperature in a vacuum oven. Then, it was kept in a desiccator and milled to produce fine particles. The same procedure was used for the synthesis of SFC using stoichiometric ratios between the NH_2 of chitosan and FFSA of 1:1, 1:2, and 1:4.

3.4 Solubility Tests of Charged Derivatives of Chitosan

Solid samples of CHI, HTACC, SCC and SFC (60 mg) were dissolved in water (20 mL) according to a method of Sashiwa *et al.* [22]. The pH of each solution was adjusted with 1.0 % aqueous HCl and NaOH.

3.5 Pre-treatment of Plasma-treated Poly(ethylene terephthalate) (Plasma-treated PET) Substrate

Plasma-treated PET substrates were soaked in aqueous solution of 1 M sodium hydroxide solution at 60 °C for 1 h. After having been incubated for a given time, the substrates were taken out, immersed in hydrochloric acid (0.1 M) for 10 min at room temperature to rinse off NaOH. Finally, the substrates were rinsed thoroughly with Milli-Q water and air-dried at room temperature to obtain the surface-hydrolyzed PET films.

3.6 Determination of Carboxyl Groups on the Plasma-treated PET Substrate

A number of carboxyl (COOH) groups on the plasma-treated PET substrate after the pretreatment was determined by a reaction with TBO according to a method of Liu *et al.* [61]. The substrate with an area of 1.54 cm^2 was immersed into a 0.5 mM TBO aqueous solution with a pH value of 10. The formation of ionic complex between the COOH groups and the cationic dye was allowed to proceed for 12 h at room temperature. The substrate was rinsed with 0.1 mM NaOH solution to remove the unbound TBO molecules. The bounded TBO on the substrate was desorbed by incubation in 4 mL 50% acetic acid solution for 10 min. The absorbance at 633 nm was recorded by a UV-vis spectrophotometer. The amount of the carboxyl groups was calculated by referring to a calibration curve of TBO/50% acetic acid solution recorded at the same conditions. The calculation is based on the assumption that 1 mol TBO has complexed exactly with 1 mol carboxyl groups.

3.7 Polyelectrolyte Self-assembly

The alternating layers were assembled by sequentially dipping the plasma-treated PET substrates in a polycation solution (2 mg/mL of PAH, or 2 mg/mL of HTACC) and a polyanion solution (1 mg/mL of SCC, 2 mg/mL of SFC, or 2 mg/mL of PAA) for 20 min interval. For most of the experiments, the salt concentration was 0.5 M NaCl in both solutions. All polyelectrolytes were not buffered. The pH of the solution was adjusted using HCl (aq) or NaOH (aq). Three pairs of polyelectrolyte self-assemblies were fabricated at pH 7 for HTACC-PAA and PAH-SCC, and at pH 8 for PAH-SFC. The substrates were rinsed thoroughly with Milli-Q water between each dipping and after the final adsorption. After the desired number of layers was deposited, the substrates were blow-dried by a light stream of nitrogen gas before contact angle analysis. To monitor the stepwise deposition of each pair of polycation-polyanion by QCM, the same procedure was also applied on 5 Hz quartz crystal coated with gold and chemically modified by a mixture of 11-mercaptoundecanoic acid and 10 mM 11-mercapto-1-undecanol. The stepwise procedure is shown in Figure 3.1.

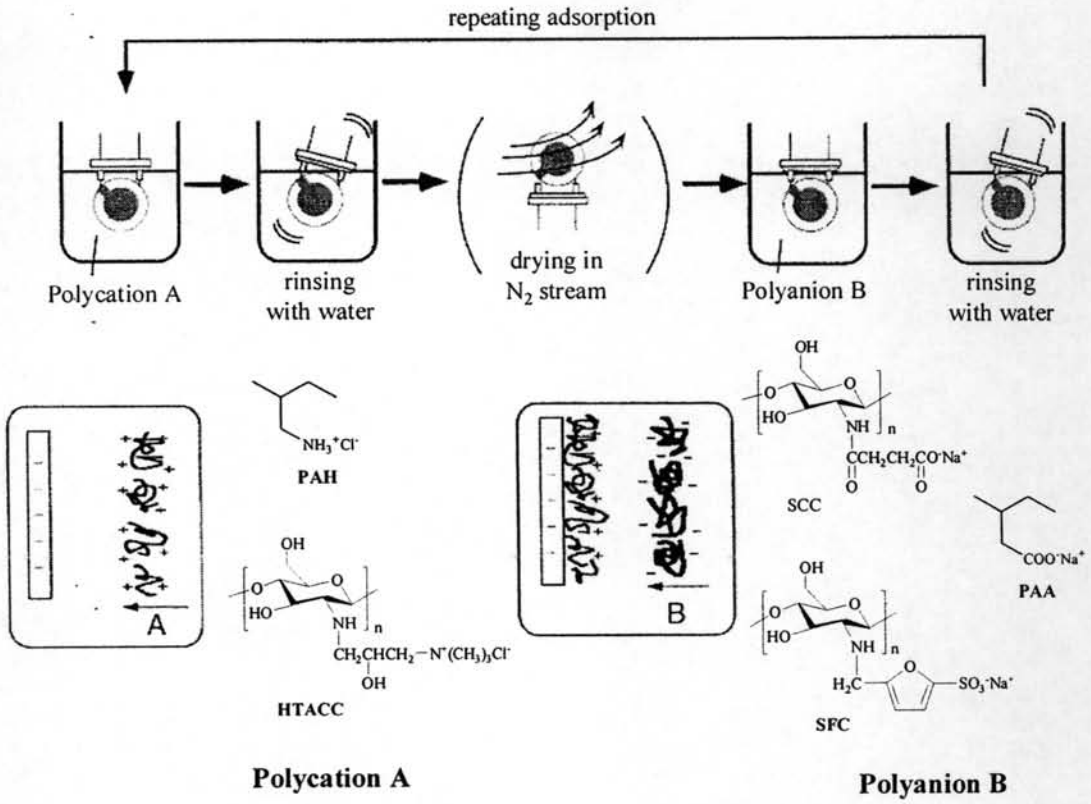

Figure 3.1 Schematic illustration of alternate layer-by-layer assembly between polycation A and polyanion B on QCM plate.

3.8 Cell Study

Fibroblast (L929) cell line was used with the density of $5x10^3$ cells/cm^2. 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/mL). They were incubated at 37°C in atmosphere containing 5% CO_2 where the culture medium was changed every 3 days.

Both the treated PET substrates with and without multilayer films were sterilized by soaking in 70% ethanol in water for 30 min and washed twice with RPMI medium 1640. The substrates were then transferred to cover the bottom of 96-well tissue culture polystyrene (TCPS) plate. Three replicated samples were used

for each condition. Approximately $5x10^3$ of L929 cells in 0.2 mL culture medium were pipetted into each well containing the substrates as well as into the bottom of 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 0.5 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 a microplate reader at the wavelength at 540 nm. Cell adhesion ratio on each surface was evaluated using Eq. (3.1) where $OD_{sample,12h}$ represents the optical density on the different polymeric surfaces and $OD_{TCPS,12h}$ represents the optical density on TCPS surfaces, which was utilized as a standard.

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

The measurement of changes in number of proliferated cells on substrates was determined at the incubation time point of 48 and 96 h. The cell proliferation ratio on each sample was evaluated using Eq. (3.2).

$$\text{Cell proliferation ratio (\%)} = \frac{OD_{sample,48\text{ or }96\,h}}{OD_{TCPS,48\text{ or }96\,h}} \times 100 \qquad (3.2)$$