CHAPTER III

EXPERIMENTAL

3.1 Materials

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

1. Chitosan, Mw = 100,000 ; 95%DD : Seafresh lab
2. 5-Formyl-2-furansulfonic acid, sodium salt (FFSA) : Aldrich
3. Triethanolamine : Fluka
4. Sodium borohydride : Fluka
5. Methanol : Merck
6. Acetone : Merck
7. Glycidyltrimethylammonium chloride (GTMAC) : Fluka
8. Acetic acid : Merck
9. Hydrochloric acid : Fluka
10. Sodium chloride : Merck
11. Ethanol : Merck
12. Poly(sodium styrene sulfonate) (PSS), M_w=70,000 : Aldrich
13. Polyacrylic acid, sodium salt (PAA), M_w=60,000 : Fluka
14. Poly(allylamine hydrochloride) (PAH), M_w=70,000 : Aldrich

15. Nanopure distilled water	: Barnstead Thermolyne Corporation
16. Bovine serum albumin (BSA)	: Aldrich
17. Lysozyme	: Aldrich
18. Fibrinogen	: Aldrich
19. Gamma-globulin	: Aldrich
20. Bicinchoninic assay kit (QuantiPro^{TM} BCA assay)	: Sigma
21. Sodium dodecyl sulfate	: Fluka
22. Phosphate buffer saline (PBS)	: Aldrich
23. Plasma-treated poly(ethylene terephthalate) film	:Wako Pure Chemical Industry, Ltd.

3.2 Equipments

3.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H NMR spectra was recorded in D_2O using 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 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were recorded with a FT-IR spectrometer (Perkin Elmer), model system 2000, with 32 scans at resolution 4 cm^{-1}. A frequency of 400-4000 cm^{-1} was collected by using TGS detector.

3.2.3 Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR)

All spectra were collected at resolution of 4 cm^{-1} and 32 scan using Bruker vector 33 FT-IR spectrometer equipped with a DTGS detector. A multiple attenuated total reflection (MATR) accessory with 45° zinc selenide (ZnSe) IRE (spectra Tech, USA) and a varible angle reflection accessory (Seagull™ , Harrick Scientific, USA) with a hemispherial ZnSe IRE were employed for all ATR spectral acquisitions.

3.2.4 Quartz Crystal Microbalance (QCM)

An AT-cut quartz crystal with a resonance frequency of 5 MHz was obtained from Maxtak, Inc. (USA) model SC-501-1. The plating crystal (1 inch in diameter) was covered by evaporated gold on both faces. The frequency was monitored by a Maxtak plating monitor (model PM-710) coupled with a MPS-550 sensor probe.

3.2.5 Contact Angle Measurements

Contact angle goniometer model Rame´-Hart 100-00 was used for the determination of water contact angles. A droplet of testing nanopure water is placed on the tested surface by bringing the surface into contact with a droplet suspended from a needle of the syringe. A silhouette image of droplet was projected on the screen and the angle is measured.

3.2.6 Microplate Reader

UV on Microplate reader, Model EL340, Bio-Tek™ Instruments Inc., was used for determining the amounts of absorbed protein using bicinconinic assay by reading UV absorbance at λ = 562 nm.

3.3 Synthesis of Charged Derivatives of Chitosan

3.3.1 *N*-Sulfofurfuryl Chitosan (SFC)

SFC was synthesized according to a modified method of Mansoor [6]. Chitosan (0.20 g, 1 equiv of NH_2) was dissolved in 1% acetic acid (10 mL) to prepare a 2.0% (w/v) solution. 10 mL of methanol containing 1.0% (w/v) triethanolamine was slowly mixed. The mixture was stirred for 6 h at room temperature. FFSA (0.16 g, 0.7 equiv) was slowly added to the chitosan slurry and the reaction proceeded for 18 hours at room temperature. As the reaction continued, the Schiff's base thus formed was reduced by slow addition of sodium borohydride (6 equiv). After reduction reaction for 6 h, the Schiff's base slowly dissolved to form a viscous solution. *N*-sulfofurfuryl chitosan 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 dessicator and milled to produce fine particles. The same procedure was used for synthesis of SFC using stoichiometric ratios of chitosan:FFSA of 1:1, 1:2 and 1:4.

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

HTACC was synthesized according to a modified method of Seong *et al.* [11]. Chitosan (0.4 g, 1 equiv of NH_2) was dissolved in 1% acetic acid to prepare a 2.0%(w/v) chitosan solution. GTMAC (0.7 g, 2 equiv) was added. 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 synthesis of HTACC using stoichiometric ratios of chitosan:GTMAC of 1:4 and 1:6.

3.4 Pretreatment of Plasma-treated Poly(ethylene terephthalate) (PET) Substrate

Plasma-treated PET substrates were soaked in sodium hydroxide solution (1M) at 60 °C for 1 h. They were then immersed in hydrochloric acid (0.1M) for 10 min at room temperature. Finally, the substrates were immersed in nanopure water for 10 min and air-dried at room temperature.

3.5 Polyelectrolyte Self-assembly

The alternating layers were assembled by sequential dipping surface-modified PET substrate in a solution of polycation (1 mg/ml of chitosan, 2 mg/ml of poly(allylamine hydrochloride) (PAH), or 3 mg/ml of HTACC) and a solution of polyanion (2 mg/ml of poly(sodium styrene sulfonate) (PSS), 2 mg/ml of SFC, or 2 mg/ml of polyacrylic acid (PAA) for 30 minute interval. 1M NaCl was added into the polymer solution. Three pairs of polyelectrolyte self-assembly were fabricated at pH 4 for chitosan-PSS, at pH 8 for PAH-SFC and at pH 7 for HTACC-PAA. Substrates were rinsed thoroughly with nanopure water between each dipping and after the final adsorption. After the desired numbers of layers were deposited, the substrates were blow-dried by a light stream of nitrogen before contact angle analysis. Stepwise deposition of each polycation-polyanion pair was monitored by QCM. The same procedure was also applied on 5Hz quartz crystal coated with gold instead of treated PET substrate.

3.6 Protocol for Protein Adsorption Test

The deposited multilayer films on treated PET substrates were placed into 24-well plate containing nanopure water in each well overnight to reach an equilibrium hydration. Each sample was removed from nanopure water and suspended into the well containing 2.0 mL protein (albumin, lysozyme, fibrinogen or γ-globulin) solution before incubated at 37 °C for 3 h. Three pieces of samples were analyzed for each condition. The samples were removed from protein solution and rinsed thoroughly with phosphate buffer saline solution (PBS) (2x) to remove any

loosely attached protein. The adsorbed protein on the sample surface was detached by soaking each sample in 2.0 mL of 1 % aqueous solution of sodium dodecyl sulfate (SDS) for 30 min. A protein analysis kit based on the bicinchoninic acid (BCA) method was used to determine the concentration of the protein dissolved in the SDS solution. 100 μL (0.1 mL) of SDS solution that soak each sample was added into designated 96 wells. 100 μL of BCA working solution was then added in each well, before the well-plate was incubated at 37°C for 2 h. The absorbance of the solution was measured at 562 nm by UV microplate reader. The amount of protein adsorbed on the samples was calculated from the protein concentration in the SDS solution. The data are expressed as mean ± standard deviation (S.D).