CHAPTER III

EXPERIMENTAL

3.1 Materials

All reagents are analytical grade and used without further purification.

1. Chitosan (DD 84.5%), M_w = 150,000 : Fluka
2. (3-Aminopropyl)triethoxysilane : Fluka
3. Poly(sodium styrene sulfonate), M_w=70,000 : Aldrich
4. Toluene anhydrous 99 % : Aldrich
5. Bovin serum albumin : Aldrich
6. Bicinchonic assay kit (QuantiPro™ BCA assay) : Sigma
7. Phosphate buffer saline (PBS) : Aldrich
8. Acetic acid : Merck
9. Toluene : Caro
10. Hydrochloric acid : Merck
11. Sodium chloride : Merck
12. Platelet- poor plasma (PPP) : Thai Red Cross Society
13. Platelet-rich plasma (PRP) : Thai Red Cross Society
14. Ultrapure distilled water : Mill-Q Lab system

15. Treated poly(ethylene terephthalate) film :Wako Pure Chemical Industry, Ltd.

3.2 Equipments

3.2.1 Ellipsometry

The ellipsometry was studied by using L115C WAFER™ ELLIPSOMETER. The thicknesses were determined in air with a 70 angle of incidence at 632.8 nm wavelength. The thickness of the adsorbed film was calculated by using the software "Dafibm" Rudolph Research, Double Absorbing Films Calculations. The calculation was based on a refractive index N_{amine} =1.421 and N_{film} = 1.500 and a silicon substrate refractive index $N_{substrate}$ = 3.858. At least five different locations on each sample were measured and the average thickness was calculated.

3.2.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectra were obtained on a thermoVGscientific using Al K_α excitation (15 Kv, 400 W). In this study, the take off angle at 70° was chosen and the approximate of depth profile is 50 Å.

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 Contact Angle Measurements

Contact angle meter model CAM-PLUS MICRO 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. A silhouette image of droplet was projected on the screen and the angle is measured.

3.2.5 Zeta-potential.

Zeta-potential model ESL 8000 (otsuka Electro, Co, Tokyo, Japan) were measured positively and negatively charged on surface. The samples were immersed in distilled water and stored at room temperature at 12 h to equilibrate the surface. The zeta-potential of sample was measured at 10 mM NaCl solution.

3.2.6 UV-Spectroscopy

UV-Spectrometer, Microtiter plate reader, Model Sunrise, Tecan Austria GmbH, was used for determining the amounts of absorbed protein using bicinconic assay by reading UV absorbance at $\lambda = 562$ nm.

3.2.7 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) Model JSM-5800L, was used to observe the morphology of surface-adherent platelets.

3.2.8 Atomic Force Microscopy (AFM)

AFM images were recorded with Atomic Force Microscope model SPI-3800, Seiko I, Tokyo, Japan. Measurements were performed in air using tapping mode. Silicon nitride tip with a resonance frequency of 13 kHz and a spring constant 0.02-0.1 N/m were used.

3.3 Preparation of Amino-containing Substrate

Silicon wafer disk was cut to 1.5x1.5 cm^2, cleaned by soaking in conc. H_2SO_4 containing $H_2Cr_2O_7$ (~3-5 wt %) and 30 % H_2O_2 in 7:3 proportion overnight, rinsed with copious amounts of deionized water and dried in a clean oven at 120 °C for 1-2 h. Amine-functionalized silicon substrate (Si-NH_2) were prepared by soaking the cleaned disks in anhydrous toluene containing 1% aminopropyltriethoxysilane under nitrogen for 8 h at room temperature, followed by rinsing in toluene (5x) and dried under vacuum.

3.4 Polyelectrolyte Self-assembly

The alternating layers were assembled by sequential dipping Si-NH_2 in 3 mg/ml poly(sodium styrene sulfonate) solution (M_w 70,000) and 0.5 mg/ml chitosan solution (M_w 150,000) for 20 min at pH 4. Substrates were rinsed with three 150 mL aliquots of 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 subjected to surface analysis. NaCl was added at the given concentrations into the polymer solution. The apparent film thickness of the resulting assemblies was measured by ellipsometer.

3.5 Photocol for Blood Compatibility Test

3.5.1 Determination of Total Amount of Adsorbed Human Plasma Protein

Human platelet-poor plasma (PPP) was obtained from the Thai Red Cross Society. The deposited multilayer films on Si-NH_2 having the dimension of 1.5 x 1.5 cm^2 were placed into 24-well tissue culture plate containing deionized water in each well. The samples were allowed to stand in the wells overnight to reach an equilibrium hydration. Each sample was removed from deionized water and suspended into the wells containing 2.5 mL PPP before incubated at 37 °C for 3 h. Three pieces of samples were analyzed for each condition. The samples were removed from PPP 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 samples in 2.5 mL of 1 % aqueous solution of sodium dodecyl sulfate (SDS) for 30 min. A protein analysis kit based on the bicinchonic 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 96-designated 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 plate 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).

3.5.2. Evaluation of Platelet Adhesion

Human platelet-rich plasma (PRP) was obtained from the Thai Red Cross Society. The deposited multilayer films on Si-NH_2 having the dimension of 1.5 x 1.5 cm^2 were placed into 24-well tissue culture plate

containing PBS in each well. The samples were allowed to stand in the wells overnight to reach an equilibrium hydration. The PRP (2.5 mL) was added into each well via a micropipet. The well plate was incubated for 1 h at 37 °C. After the PRP was removed using a micropipet, the substrates were rinsed with PBS (3x). The saline solution containing 2.5 % (v/v) glutaraldehyde was added to each well in order to fix the platelets adhered on the sample surfaces. The samples were rinsed with PBS (3x) followed by deionized water (3x) prior to dehydration by sequentially soaking in 30, 50, 70, 90, 99 and 100 % (v/v) ethanol in water for a period of 10 min interval. The samples were dried under vacuum for 24 h then sputtered with gold before analyzed by scanning electron microscopy (SEM).