CHAPTER III

EXPERIMENTAL

3.1 Materials

All reagents and materials are analytical grade

1. Acetic acid : Merck
2. Acetone : Merck
3. Allyl alcohol : Merck
4. Bicinchoninic assay kit (QuantiPro™ BCA assay) : Sigma
5. Bovine serum albumin (BSA) : Aldrich
6. 2-Bromo-2-methylpropionyl bromide : Fluka
7. *tert*-Butyl acrylate (*t*-BA) : Aldrich
8. Chitosan, Mw = 100,000 ; 96%DD : Seafresh Lab Co. Ltd.
9. Chloroform : Merch
10. Copper (I) bromide : Fluka
11. Dichloromethane : Merck
12. Dimethylchlorosilane : Gelest
13. *Escherichia coli (E.coli)* : National Center for Genetic Engineering and Biotechnology (Biotec)
14. Ethanol : Merck
15. Glycidyltrimethylammonium chloride (GTMAC) : Fluka
16. Heparin : BioChemika
17. Hexane : Merck
18. Hydrochloric acid : Fluka
19. Hydrogen peroxide : Univar
20. *N, N, N', N'', N''*-pentamethyldiethylenetriamine : Aldrich
21. 10,12-Pentacosadiynoic acid (PCDA) : Fluka
22. Phosphate buffer saline (PBS) : Aldrich
23. Platinum on activated Charcoal : Fluka

24. Poly(acrylic acid), sodium salt (PAA), M_w=60,000 : Fluka
25. Propanol : Univar
26. Silica gel 60 (0.063-0.200 mm) : Merck
27. Silicon wafer (single sided and double sided) : Siltron Inc. Korea
28. Sodium chloride : Merck
29. Sodium hydroxide : Fluka
30. Sodium dodecyl sulfate (SDS) : Fluka
31. Sodium sulfate anhydrous : Fluka
32. Sulfuric acid : Merck
33. Tetrahydrofuran : Carlo
34. Toluene : Carlo
35. Triethylamine : Carlo
36. Trifluoroacetic acid : Fluka
37. Triplicate soy agar (TAB) : PPL system Co., Ltd.
38. Triplicate soy broth (TSA) : PPL system Co., Ltd.
39. Toluene anhydrous 99 % : Aldrich
40. Milli-Q water : Mill-Q Lab system

3.2 Equipments

3.2.1 Ellipsometry

The ellipsometry was studied by using Gaertner Ellipsometer L117. The thicknesses were determined in air with a 70° of incidence angle at 632.8 nm. The calculation was based on a refractive index; $N_{initiator}$ = 1.443, $N_{t\text{-}BA}$ = 1.460, $N_{hydroxyl}$ = 1.462 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 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^1H NMR spectra were recorded in $CDCl_3$ or D_2O using a 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.

3.2.3 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. The sample containing silica particles were prepared as KBr pellets.

3.2.4 Contact Angle Measurement

Contact angle goniometer model Ramé-Hart 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. The measurements were carried out in air at the room temperature. 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. 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.

3.2.5 Atomic Force Microscopy (AFM)

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

3.2.6 Gel Permeation Chromatography (GPC)

The molecular weight and molecular weight distributions of the poly(*t*-butyl acrylate) (P*t*-BA) were determined by gel permeation chromatography (GPC) using THF as eluent, Water E600 column connected to the RI detector. The flow rate was 1 mL/min. Narrow PS standards were used for the calibration curve. In case of the poly(acrylic acid) (PAA), aqueous gel permeation chromatogram obtained from

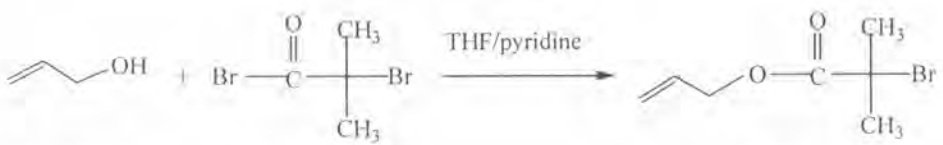
Water 600 controller chromatograph, connected to the RI detector, equipped with ultrahydrogel linear and guard column at 30 °C. Water was used as eluent with the flow rate of 0.6 mL/min. Poly(ethylene glycol) was used as standards for calibration.

3.2.7 UV-Vis Spectroscopy

UV-Vis spectroscopy Model Techna, specgene was used for determination the amount of carboxylic groups of poly(acrylic acid) brushes on surface by reading the absorbance at 633 nm.

3.3 Synthesis of α-Bromoester Derivatives to be used as Initiators

3.3.1 Synthesis of 2-Bromo-2-methylpropionic acid allyl ester (1)



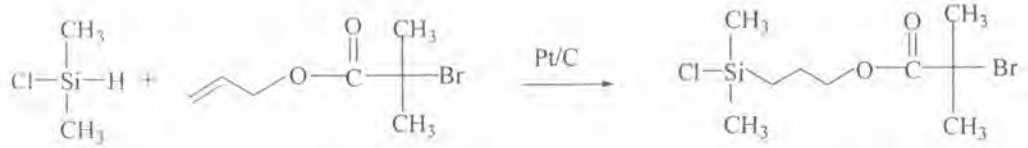
allyl alcohol — 2-Bromo-2-methylpropionyl bromide — 2-Bromo-2-methylpropionic acid allyl ester

(1)

To a solution of allyl alcohol (1.70 mL, 25 mmol) in 25 mL of dry tetrahydrofuran, pyridine 2.1 mL (26.5 mmol) was added, followed by a dropwise addition of 2-bromo-2-methylpropionyl bromide (3.10 mL, 25 mmol). The mixture was stirred at room temperature overnight, diluted with hexane and then washed once with 2N HCl and twice with deionized water. The organic phase was dried over sodium sulfate and filtered. After the solvent was removed from the filtrate under reduced pressure, the colorless oily residue was purified by filtering through a silica gel column chromatography to give the product in 90% yield.

^{1}H NMR ($CDCl_3$) of (1): δ 1.98 (6H, $C(C\underline{H}_3)_2$, s), 4.71 (2H, $OC\underline{H}_2$, d, J = 5.46 Hz), 5.30-5.44 (2H, $=C\underline{H}_2$, complex m), 5.93-6.0 (1H, $=C\underline{H}$, complex m).

3.3.2 Synthesis of 2-Bromo-2-methylpropionic acid 3-(dimethylchloro silanyl) propyl ester (2)



Dimethylchloro silane

2-Bromo-2-methylpropionic acid allyl ester

(1)

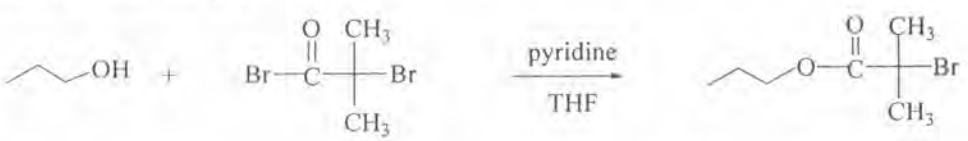
2-Bromo-2-methylpropionic acid 3-(dimethylchlorosilanyl)propyl ester

(2)

Allylic ester **(1)** (2 mL, 12 mmol) was mixed with 20 mL of freshly distilled dimethylchlorosilane in 50 mL round bottom flask equipped with a condensor. Pt/C (10% Pt) (20 mg) were added and the mixture was refluxed for 15 h. The excess chlorosilane was removed under reduced pressure. The oil residue was filtered through anhydrous sodium sulfate to remove the catalyst to give chlorosilane initiator **(2)** as a light-yellow oil. (76% yield)

^{1}H NMR ($CDCl_3$) of (2): δ 4.15-4.21 (2H, $C\underline{H}_2O$), s), 1.94 (6H, $C(C\underline{H}_3)_2$), 1.65-1.80 (2H, $-C\underline{H}_2-$), 0.61 (2H, $-SiC\underline{H}_2-$), 0.04 (6H, $-Si(C\underline{H}_3)_2-$).

3.3.3 Synthesis of 2-Bromo-2-methylpropionic acid propyl ester as a "Sacrificial" Initiator (3)



Propanol

2-Bromo-2-methylpropionyl bromide

2-Bromo-2-methylpropionic acid propyl ester

(3)

To a solution of propanol (1.5 g, 25 mmol) in 25 mL of tetrahydrofuran, pyridine (3.1 mL, 26.5 mmol) was added, followed by a dropwise addition of 2-bromoisobutyryl bromide (3.10 mL, 25 mmol). The mixture was stirred at room temperature overnight, diluted with hexane and washed once with 2N HCl and twice with deionized water. The organic phase was dried over sodium sulfate and filtered. After the solvent was removed from the filtrate under reduced pressure, and the

colorless oily residue was purified by filtering through a silica gel column chromatography to give the desired product in 92% yield.

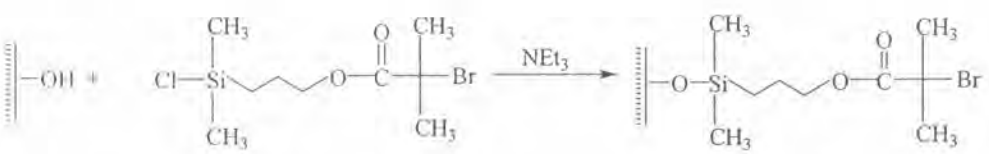
^{1}H NMR ($CDCl_3$) of (3): δ 1.0 (3H, $OCH_2CH_2C\underline{H}_3$, t, J = 7.02 Hz), 1.72 (2H, $OCH_2C\underline{H}_2CH_3$, complex m), 1.95 (6H, $C(C\underline{H}_3)_2$, s), 4.15 (2H, $OC\underline{H}_2CH_2CH_3$, t, J = 6.24 Hz).

3.4 Preparation of Polymer Brushes

3.4.1 Pretreatment of Silicon Substrates

Silicon wafers were cut into 1.1 x 1.1 cm^2 substrates. The substrates held in a slotted hollow glass cylinder (custom designed holder) were put in a freshly prepared mixture of 7 parts of concentrated sulfuric acid and 3 parts of 30% hydrogen peroxide. Substrates were submerged in the solution at room temperature for 2 h, rinsed with five to seven aliquots of deionized water and placed in a clean oven at 120°C for 2 h. Silanization reaction was carried out immediately after treating the substrates in this fashion.

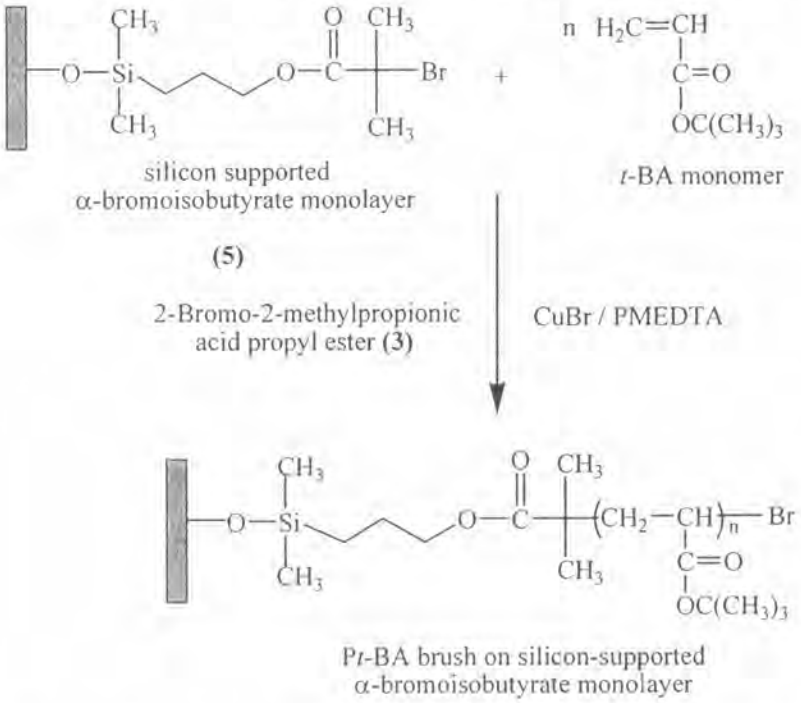
3.4.2 Preparation of Surface-tethered Initiator (5)



(2) **(5)**

Freshly cleaned silicon substrates held in a slotted hollow glass cylinder were placed into a dried Schlenk flask. Anhydrous toluene (30 mL) containing triethylamine (60 μL, 0.43 mmol) was transferred to the flask *via* cannula. 2-Bromo-2-methylpropionic acid 3-(chlorodimethylsilanyl)propyl ester **(2)** (100 μL, 0.1225 g, 0.41 mmol) was then added by a syringe. Reactions were carried out under nitrogen atmosphere at ambient temperature for 18 h. The substrates were rinsed with 1x10 mL of toluene, 2x10 mL of 2-propanol, 2x10 mL of ethanol, 1x10 mL of ethanol-water (1:1), 1x10 mL of water and 1x10 mL of ethanol and dried under vacuum.

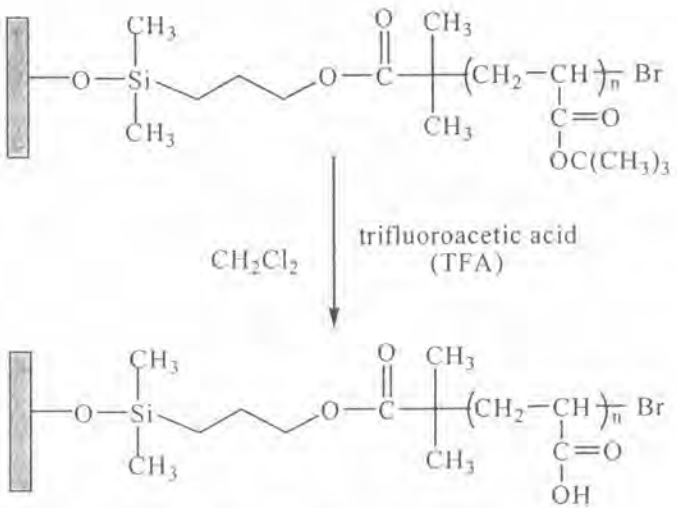
3.4.3 Surface-initiated Polymerization of *tert*-Butyl Acrylate



The silicon substrates bearing ∝-bromoisobutyrate monolayer **(5)** held in a slotted hollow glass cylinder were placed in a Schlenk flask and sealed with a rubber septum. The flask was evacuated and back-filled with nitrogen three times and left under a nitrogen atmosphere. CuBr (0.0493 g, 0.34 mmol), *t*-BA monomer (10 mL, 68 mmol), and acetone (15 mL) were added to a separate Schlenk flask with a magnetic stirrer bar, sealed with a rubber septum, and degassed by purging with nitrogen for 1 h. PMDETA (71.8 μL, 0.34 mmol) was added to the mixture via a syringe, and the solution was stirred at 60°C until it became homogeneous (approximately 5 min). The solution was then transferred to the flask containing the silicon substrates via cannula, followed by the addition of "sacrificial" initiator 2-bromo-2-methylpropionic acid propyl ester **(3)** (0.0718 mg, 0.34 mmol) via syringe. The polymerization was allowed to proceed at 60°C. After a set reaction time, the silicon substrates were removed and rinsed with THF. To remove untethered polymer, the silicon substrates were placed in a Soxhlet extractor and extracted with THF for 24 h and dried under vacuum. The substrates bearing poly(*tert*-butyl acrylate) (P*t*-BA) brushes were then analyzed by contact angle measurements and ellipsometry. Free P*t*-BA from the solution was isolated by first evaporating residual

monomer and solvent under reduced pressure and dissolving in THF. The polymer solution in THF was then past through a short column of silica to remove any residual catalyst and analyzed by GPC.

3.4.4 Preparation of Surface-tethered Poly(acrylic acid) (PAA) Brushes by Hydrolysis of Poly(*tert*-butyl acrylate) Brushes



poly(acrylic acid) brushes

The silicon substrates bearing P*t*-BA brushes held in a slotted hollow glass cylinder were placed in Schlenk flask. The mixture of trifluoroacetic acid (2.9 mL, 37.5 mmol) and dichloromethane (15 mL) were added and stirred at room temperature for 6 h. The substrates were removed, rinsed with copious amount of dichloromethane, and then dried under vacuum.

3.5 Determination of Carboxyl Group Density of Surface-tethered Poly(acrylic acid) Brushes

The toluidine blue o staining method was employed to determine the amount of carboxyl groups on PAA brushes. A 0.5 mM dye aqueous solution was prepared at pH 10. The silicon substrates bearing PAA brushes were placed in the dye solution for 6 h at 30°C. The substrates were then removed and thoroughly washed with a sodium hydroxide solution of pH 9 for 24 h to remove any noncomplexed dye

adhering to the substrates. The dye that was complexed with carboxyl groups was desorbed from the surface by soaking the substrates in a 50% acetic acid solution for 16 h. The desorbed dye content was obtained by measuring of the optical density of the solution at 633 nm with an UV-vis spectrophotometer. The PAA content was obtained from a calibration plot of the optical density versus dye concentration assuming one carboxyl group reacted with one dye molecule.

3.6 Synthesis of *N*-[(2-Hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC)

HTACC was synthesized according to a method of Seong *et al.* [82]. 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 (1.4 g, 4 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.

^{1}H NMR (D_2O) of HTACC: δ 4.85, 3.15, 3.5-4.0 (the proton of C1, C2 and C3-6 of glucosamine in chitosan), 2.85 (2H, $NC\underline{H}_2CH(OH)CH_2$), 3.38 (2H, $NCH_2CH(OH)C\underline{H}_2$), 4.60 (1H, $NCH_2C\underline{H}(OH)CH_2$), 3.1 (9H, $N(C\underline{H}_3)_3$).

3.7 Preparation of Poly(10,12-pentacosadiynoic acid) (PPCDA) vesicles

PPCDA was synthesized according to a method of Potisatityuenyong *et al.* [77]. The diacetylene monomer 10,12-Pentacosadiynoic acid (PCDA, 11 mg) as a white solid was dissolved in diethyl ether (2 mL) in test tube and filtered to remove any contaminating polymerized materials. The filtrate was dried under a rotary evaporator in the dark to produce a thin film of lipid monomer. Milli-Q water was added to provide a 1 mM aqueous lipid suspension and was sonicated at 75-80 °C for 30 min. the vesicle suspension was allowed to cool at room temperature and then kept at overnight. The vesicle suspension was irradiated by UV (254 nm) irradiation for 5 min to complete the topopolymerization. Following the polymerization, the solution was filtered through a 0.45-μm PTFE syringe filter to remove any undesired lipid aggregates formed during the preparation. The resulting filtrate appeared as a

transparent deep blue solution of poly(10, 12-pentacosadiynoic acid) (PPCDA) vesicles.

3.8 Multilayer Assembly on Surface-tethered Poly(acrylic acid) Brushes

Layer-by-layer deposition was carried out by alternatively dipping the surface-tethered PAA brushes into 1 mg/mL of chitosan and 1 mg/mL of PAA at pH 4 or 1 mM PPCDA at pH 5.6 for 40 min interval. The substrates were rinsed thoroughly with Milli-Q water three times after each deposition step. When the desired PAA/CHI mutilayer was achieved, heparin or *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) was last deposited as a top layer. After the final deposition, the substrates were dried under stream of nitrogen and dried under vacuum.

3.9 Bioactivity of the Multilayer Film Deposited on Surface-tethered Poly(acrylic acid) Brushes

3.9.1 Protocol for Protein Adsorption Test

The deposited multilayer films on PAA brushes substrates were placed into 24-well plate containing Milli Q water in each well overnight to reach an equilibrium hydration. Each sample was removed from Milli Q water and suspended into the well containing 2.0 mL albumin 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 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 SDS for 30 min. A protein analysis kit based on the 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 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).

3.9.2 Protocol for Antibacterial Activity Test

Triplicate soy broth (TSB) was used as a growth medium for the antibacterial assays, *Escherichia coli* was used as *gram negative* bacterium. All glasswares used for the tests were sterilized in an autoclave at 121°C for 15 min prior to use. The multilayer films on glass samples were sterilized by exposing to UV light for 30 min prior to the tests.

The deposited multilayer films on PAA brushes substrates (1.1x1.1 cm^2) was placed in a well of a 24-well plate containing 2 mL TSB. A 12 μL of bacterial suspension in distilled water (OD_{600} is equal to 0.50) was pipetted into the well. The well plate was placed in an incubator shaker operated at 110 rpm, 37°C. Then, the bacterial suspension (100 μL) was pipetted from the well plate and placed in a well of a 96-well plate to determine OD_{600} by UV-Vis spectroscopy. Another 100 μL of the bacterial suspension was diluted to 10^{10} times. A 100 μL of the diluted bacterial suspension was then spreaded onto the triplicate solid agar using the spread plate method. After incubating at 37°C for 18 h, a number of viable bacteria were then counted. The results after multiplication with the dilution factor were expressed as mean colony forming units per volume (CFU/mL).