CHAPTER III

EXPERIMENTAL

3.1 Materials

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

1. Allyl alcohol : Merck
2. Ammonium chloride : Merck
3. Bicinchonic assay kit (QuantiPro™ BCA assay) : Sigma
4. 2, 2'-Bipyridyl : Fluka
5. 2-Bromoisobutyryl bromide : Fluka
6. Copper (I) bromide : Fluka
7. Dichloromethane : Merck
8. Diethyl ether : Carlo
9. Dimethoxyethane : Fluka
10. Dimethylethoxysilane : Gelest
11. Ethanol : Merck
12. Ethyl acetate : Merck
13. Hexane : Merck
14. Hydrogen hexachloroplatinate (IV) hexahydrate : Aldrich
15. Hydrogen peroxide : Univar

16. Isopropyl alcohol : Merck

17. Magnesium sulfate anhydrous : Unilab

18. 2-Methacryloyloxyethyl phosphorylcholine : NOE corporation, Japan

19. Methanol : Merck

20. Phosphate buffer saline (PBS) : Aldrich

21. Platelet-poor plasma (PPP) : Thai Red Cross Society

22. Platelet-rich plasma (PRP) : Thai Red Cross Society

23. Poly(ethylene glycol) 350 monomethyl ether : Fluka
(OEG; mean degree of polymerization is 7-8)

24. 1-Propanol : Univar

25. Protein standard (Bovine Serum Albumin: BSA): Sigma

26. Silica gel 60 (0.063-0.200 mm) : Merck

27. Silicon wafer (Single-sided, polished) : Siltron Inc. Korea

28. Silicon wafer (Double-sided, polished) : Siltron Inc. Korea

29. Sodium dodecyl sulfate : Fluka

30. Sodium sulfate anhydrous : Fluka

31. Sulfuric acid : Merck

32. Tetrahydrofuran : Carlo

33. Toluene : Carlo

34. Triethylamine : Carlo

35. Toluene anhydrous 99 % : Aldrich

36. Ultrapure distilled water : Mill-Q Lab system

3.2 Equipment

3.2.1 Ellipsometry

The ellipsometry was studied by using L115C WAFER^{TM} ELLIPSOMETER. The thicknesses were determined in air with a 70° of incidence angle at 632.8 nm. The thickness of the adsorbed film was calculated using the software "Dafibm" Rudolph Research, Double Absorbing Films Calculations. The calculation was based on a refractive index $N_{initiator}$ = 1.443, N_{MPC} = 1.488, N_{MMA} = 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 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 75° were chosen and the approximation of depth profile is ~10 and ~40 Å, respectively.

3.2.3 Nuclear Magnetic Spectroscopy (NMR)

The ^{1}H NMR spectra and ^{13}C NMR spectra were recorded in either $CDCl_3$, D_2O or CD_3OD 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.

3.2.4 Gel Permeation Chromatography (GPC)

The molecular weight and molecular weight distributions of the MPC homopolymers were determined by aqueous gel permeation chromatography (GPC), using Shodex Ohpak SB-803 HQ column connected to the RI detector. The flow rate was 0.5 mL/min. The eluent was water including 10 mM LiBr. Calibration was based on poly(ethylene glycol)(PEG) standards ranging from 6,000 to 50,000 g mol^{-1}.

3.2.5 Freeze Dryer

Freeze dryer model Freezone 77520, Benchtop, Labconco was used to dry the MPC homopolymers.

3.2.6 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 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 areas of each sample.

3.2.7 UV-Spectroscopy

UV on Microtiter plate reader, model Sunrise, Tecan Austria GmbH, was used for determining the amounts of the absorbed protein using bicinconic assay by reading UV absorbance at λ = 562 nm.

3.2.8 Scanning Electron Microscopy (SEM)

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

3.2.9 Atomic Force Microscopy (AFM)

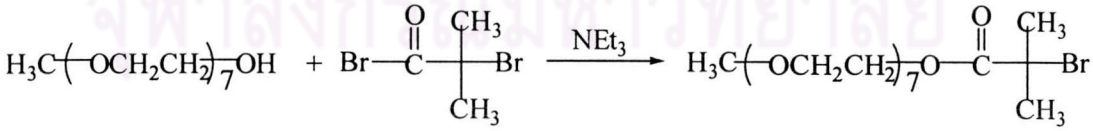
AFM images were recorded with Atomic Force Microscope model SPI-3800, Seiko I, Tokyo, Japan. Measurements were performed in air using tapping mode. Silicon nitride tip with a resonance frequency of 13 kHz and a spring constant 0.02-0.1 N/m were used.

3.2.10 Atomic Absorption (AA)

Atomic adsorption (AA) model AAnalyst 100 Perkin-Elmer was used to analyze the residual Cu quantity of the purified MPC homopolymer.

3.3 Synthesis of PMPC in solution

3.3.1 Synthesis of Methoxy-capped Oligo(ethylene glycol)-2-bromoiso butyrate initiator : OEGBr (1)



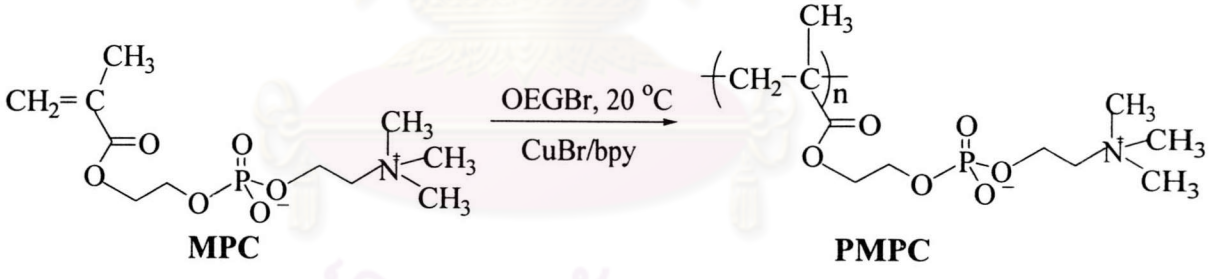
1

In a three-necked flask, 2-bromoisobutyryl bromide (2.09 g, 10 mmol) and triethylamine (0.71 g, 10 mmol) were mixed in tetrahydrofuran (6 mL) at 0°C. An 8.57 mL aliquot of a 33% (w/v) tetrahydrofuran solution of poly(ethylene glycol) 350 monomethyl ether: OEG (2.83 g, 6.57 mmol) was added dropwise. After the

addition was complete, the reaction solution was allowed to warm to room temperature, and the reaction mixture was stirred for another 4 h. The reaction mixture was then filtered, and tetrahydrofuran was removed using the rotary evaporator. The resulting yellow crude product was dissolved in water and extracted with diethyl ether. The diethyl ether layer was collected and dried over $MgSO_4$. The diethyl ether was removed under vacuum to give methoxy-capped oligo(ethylene glycol)-2-bromoisobutyrate: OEGBr **(1)** as an orange viscous liquid (2.79 g, 80 %yield).

^{1}H NMR ($CDCl_3$): δ 1.88 (6H, $C(C\underline{H}_3)_2$, s), 3.32 (3H, $OC\underline{H}_3$, s), 3.50 (2H, $(CO)OCH_2C\underline{H}_2$, t, J = 3.90 Hz), 3.59 (24H, $OC\underline{H}_2$, s), 3.69 (2H, $CH_3OC\underline{H}_2$, t, J = 3.90 Hz), 4.27 (2H, $(CO)OC\underline{H}_2$, t, J = 3.90 Hz).

3.3.2 Synthesis of PMPC



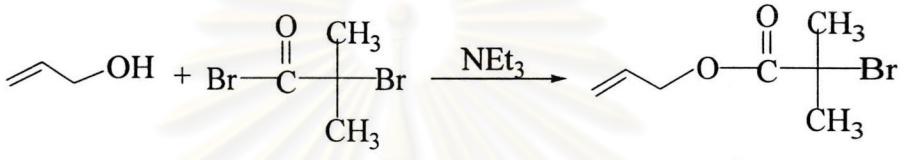
MPC **PMPC**

The water-soluble ATRP initiator **(1)** (67.0 mg, 0.135 mmol, 1 equiv) was dissolved in Milli-Q deoxygenated water (10 mL). After the mixture was degassed by one freeze-pump-thaw cycle and purged with nitrogen for 30 min, Cu(I)Br catalyst (19.0 mg, 0.135 mmol, 1 equiv) and bpy ligand (42.0 mg, 0.27 mmol, 2 equiv) were added to the stirred solution under nitrogen. MPC (0.8 g, 2.7 mmol, 20 equiv, target M_n = 6,000) was then added as a solid to the reaction mixture under nitrogen. The polymerization was allowed to proceed for the set reaction time. The MPC homopolymer was precipitated into tetrahydrofuran, then redissolved in water, and passed through a silica gel column to remove residual ATRP catalyst.

Polymerization in distilled methanol, distilled 2-propanol or mixed solvent was carried out using the same protocol.

3.4 Preparation of Surface Grafted ∝-bromoester Initiators

3.4.1 Synthesis of Prop-2'-enyl 2-bromo-2-methylpropinoate (2)

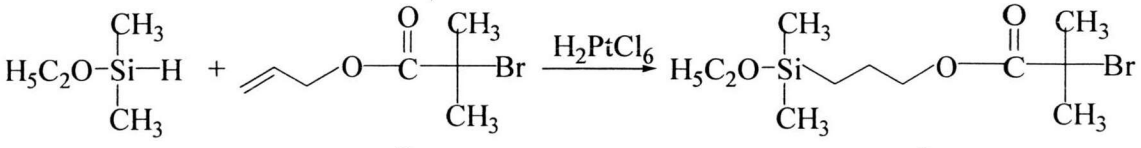


2

2-Bromoisobutyryl bromide (11.5 g, 50 mmol) was added in a dropwise fashion to a stirred solution of allyl alcohol (2.90 g, 50 mmol) and triethylamine (5.06 g, 50 mmol) in dry dichloromethane (30 mL). After stirring at 0 °C under nitrogen for 1 h, the reaction mixture was allowed to warm to room temperature and stirred for an additional 2.5 h. The precipitated triethylamine hydrochloride was removed by filtration, and the solution was washed with aqueous ammonium chloride (saturated) and water. The dichloromethane layer was collected and dried over $MgSO_4$, and the dichloromethane was then removed using the rotary evaporator to give prop-2'-enyl 2-bromo-2-methylpropinoate: **(2)** as a pale yellow viscous liquid (9.31 g, 90 %yield).

^{1}H NMR ($CDCl_3$): δ 1.94 (6H, $C(C\underline{H}_3)_2$, s), 4.66 (2H, $OC\underline{H}_2$, d, J = 5.46 Hz), 5.25-5.40 (2H, $=C\underline{H}_2$, complex m), 5.88-5.98 (1H, $=C\underline{H}$, complex m).

3.4.2 Synthesis of 3-(Dimethylethoxysilyl)propyl-2-bromoisobutyrate (3)



2 **3**

To a solution of the alkene derivative **(2)** (2.07 g, 10 mmol) in dimethylethoxysilane (1.2 mL, 10 mmol) a 1:1 ethanol/dimethoxyethane solution of chloroplatinic acid, H_2PtCl_6 (1.1 mg, 0.2 mL) was added. The reaction mixture was stirred at room temperature under nitrogen in the dark for 14 h. Dry toluene (3 mL) was then added and the excess dimethylethoxysilane was removed under reduced pressure. Dry dichloromethane was added and then removed under reduced pressure. The crude product was passed through a short column of dry sodium sulfate, the column was washed with dry dichloromethane and the dichloromethane was removed under reduced pressure to give the desired product as a yellow viscous liquid **(3)** (2.17 g, 70 %yield).

^{1}H NMR ($CDCl_3$): δ 0.04 (6H, $Si(C\underline{H}_3)_2$, s), 0.931 (2H, $OCH_2CH_2C\underline{H}_2$, t, J = 7.04 Hz), 1.20 (3H, $SiOCH_2C\underline{H}_3$, t, J = 7.04 Hz), 1.66 (2H, $OCH_2C\underline{H}_2CH_2$, complex m), 1.90 (6H, $C(C\underline{H}_3)_2$, s), 3.69 (2H, $SiOC\underline{H}_2CH_3$, q, J = 7.04 Hz), 4.09 (2H, $OC\underline{H}_2CH_2CH_2$, t, J = 7.04 Hz).

3.4.3 Preparation of silicon oxide substrates

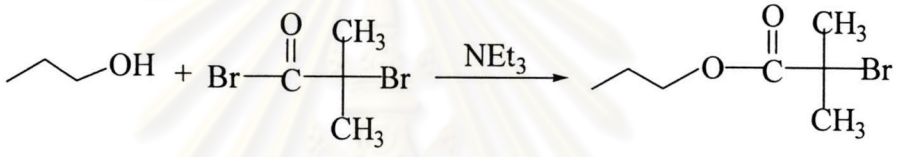
The single-sided polished silicon wafers were cut to 1.5x1.5 cm^2 and soaked for 2 h in 7:3 (v/v) concentrated sulfuric acid and hydrogen peroxide. Then the wafers were rinsed extensively with deionized water and dried in an oven at 120 °C for 2 h. Double-side polished silicon wafers were cleaned similarly and used as substrates for protein adsorption analysis.

3.4.4. Preparation of Surface Grafted α-bromoester Initiators (4)

3 **4**

Surface-tethered α-bromoester **(4)** was prepared by soaking the cleaned silicon oxide substrates in anhydrous toluene containing 2 mM (24.8 mg in 40 mL) of 3-(dimethylethoxysilyl) propyl-2-bromoisobutylrate **(3)** under nitrogen at ambient temperature. After a certain period of time, the substrates were removed and rinsed by toluene (3x), ethanol (3x), 1:1 ethanol:H_2O (2x), DI water (2x), ethanol (2x) and DI water (3x), respectively and dried under vacuum.

3.4.5 Synthesis of Prop-2-bromo-2-methylpropinoate (5)

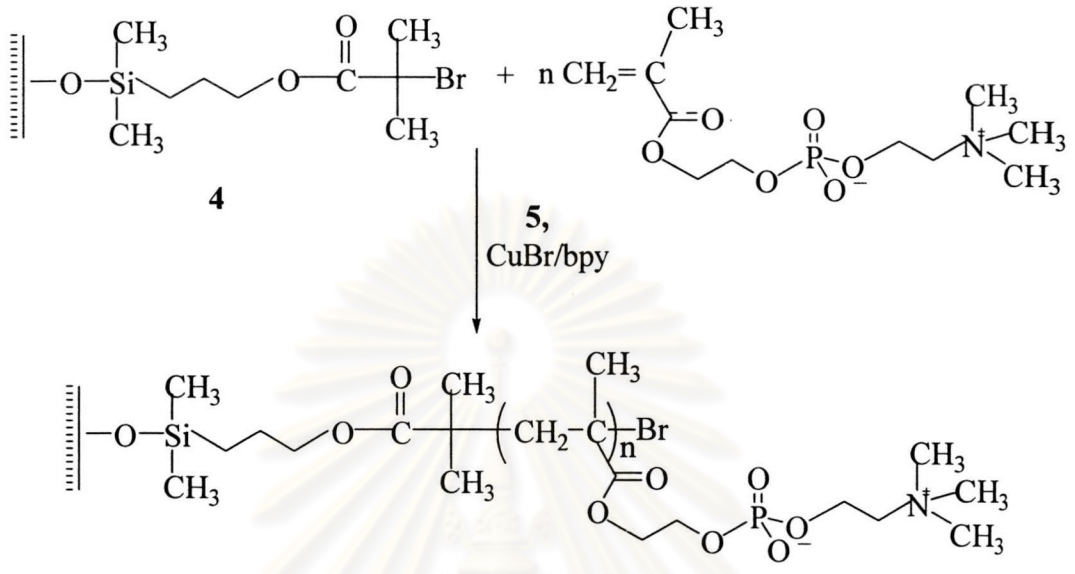


5

2-Bromoisobutyryl bromide (11.5 g, 50 mmol) was added in a dropwise fashion to a stirred solution of 1-propanol (3.01 g, 50 mmol) and triethylamine (5.06 g, 50 mmol) in tetrahydrofuran (20 mL). After stirring at 0 °C under nitrogen for 1 h, the reaction mixture was allowed to warm to room temperature and was stirred for an additional 4 h. The precipitated triethylamine hydrochloride was removed by filtration, and the tetrahydrofuran was removed using the rotary evaporator. The resulting yellow crude product was dissolved in water and extracted with diethyl ether, aqueous ammonium chloride (saturated) and water. The solution was dried over $MgSO_4$. The solvent was removed under vacuum to give prop-2-bromo-2-methylpropinoate **(5)** as a pale yellow viscous liquid (9.40 g, 90 %yield).

^{1}H NMR ($CDCl_3$): δ 0.98 (3H, $OCH_2CH_2C\underline{H}_3$, t, J = 7.62 Hz), 1.71 (3H, $OCH_2C\underline{H}_2CH_3$, m), 1.93 (6H, $C(C\underline{H}_3)_2$, s), 4.14 (2H, $OC\underline{H}_2CH_2CH_3$, d, J = 6.45 Hz).

3.5 Preparation of PMPC Brushes



All solvents were distilled, degassed by two freeze-pump-thaw cycles. The substrates having surface-tethered α-bromoester **(4)** were placed in a Schlenk flask containing CuBr (4.75 mg, 0.033 mmol), bpy (10.5 mg, 0.067 mmol), prop-2-bromo-2-methylpropionate **(5)** (3.5 mg, 0.017 mmol), and glass wool, 15 mL of degassed solvent was then added via cannular under nitrogen atmosphere and purged with nitrogen for 30 min before use. The MPC monomer (0.2 g, 0.67 mmol) was dissolved in 15 mL of degassed solvent and then transfer via cannular under nitrogen atmosphere into a Schlenk reaction flask. The polymerization was allowed to proceed at ambient temperature. After a set reaction time, the polymerization was stopped by precipitating the solution into tetrahydrofuran to obtain PMPC formed from the "added" initiator (prop-2-bromo-2-methylpropinoate **(5)**) which was analyzed by GPC. The substrates were then removed and washed by the solvent used for polymerization, soxhlet extracted with methanol overnight and dried under vacuum. The substrates bearing polymer brushes were then analyzed by XPS, contact angle measurement, ellipsometry and AFM.

3.6 Protocol for Blood Compatibility Test

3.6.1 Determination of Total Amount of Adsorbed Human Plasma Protein

Human platelet-poor plasma (PPP) was obtained from the Thai Red Cross Society. The substrates bearing polymer brushes having the dimension of 1.5 x 1.5 cm^2 were placed into a 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 in the wells containing 3.0 mL PPP before being 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 sample in 3.0 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).

3.6.2 Evaluation of Platelet Adhesion

Human platelet-rich plasma (PRP) was obtained from the Thai Red Cross Society. The substrates bearing polymer brushes having the dimension of 1.5 x 1.5 cm^2 were placed in 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 (3.0 mL) was added into each well by 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 an interval of 10 min. The samples were dried under vacuum for 24 h then sputtered with gold before analyzed by scanning electron microscopy (SEM).