# CHAPTER II

# EXPERIMENTAL

## 2.1 Materials

Dichloromethane (DCM) was distilled over $CaH_2$. Methanol and *N, N* dimethylsulfoxide (DMSO) were distilled over molecular sieve type 4A. Oxalyl chloride, sodium cyanoborohydride were purchased from Fluka Chemika. Bovine serum albumin, lysozyme, bicinchoninic assay kit, phosphate buffer saline (PBS), and triethylamine (TEA) were purchased from Aldrich Chemical Co. Chitosans $M_v$ = 645,535 was obtained from Seafresh Chitosan (Lab) Co., Ltd. Monomethoxy Poly (ethylene glycol) (MPEG) Mw = 550 daltons and monomethoxy triethylene glycol (MTEG) were obtained from Fluka Chemika. Butyraldehyde was obtained from Merck.

## 2.2 Equipment

### 2.2.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra of chitosan were obtained from 1% chitosan dissolved in 1% $CD_3COOD$ in $D_2O$ using 400 MHz ($^1H$) on Varian mercury-400 spectrometer. Chemical shifts are in ppm.

### 2.2.2 Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectra were recorded on a Nicolet Impact 410 Spectrophotometer. The sample was prepared as a potassium bromide (KBr) pellet by mixing the polymer with KBr and pressed into a pellet.

### 2.2.3 Attenuated total reflectance infrared (ATR-IR) spectroscopy

All spectra were collected at resolution of 4 $cm^{-1}$ and 64 scan co-addition 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 variable angle reflection accessory (Seagull™, Harrick Scientific, USA) with a hemispherical ZnSe IRE were employed for all ATR spectral acquisitions.

### 2.2.4 Air–water contact angle measurement

Model CAM-PULS MICRO is designed to measure contact angles on very small areas of a variety of surfaces, flat, curved and cylindrical, such as small-diameter tubings. It is important however, that the measured surface is smooth and homogeneous. A droplet of testing liquid is placed on the tested surface by bringing the surface into contact angle with a droplet suspended from a needle of the syringe.

1. Platform
2. Specimen holder
3. Knurled knob
4. Focus range
5. Micrometer syringe
6. fiber optic illuminator
7. Projection lens
8. Inversion prism
9. Plate
10. Circular screen

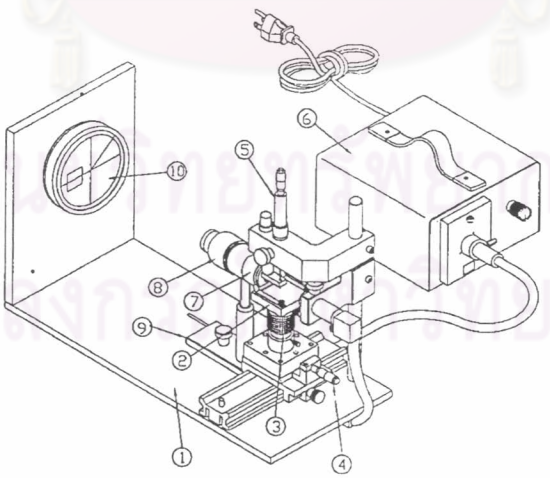


**Figure 2.1** Instrument set up for the measurement of air-water contact angle

### 2.2.5 UV-spectroscopy

UV-spectrometer, microtiter plate reader; model Sunrise, Tecan Austria GmbH, was used for determining the amounts of adsorbed protein at a wavelength of 562 nm.

## 2.3 Preparation of chitosan films

Chitosan (2 g) was dissolved in 0.1 M acetic acid (100 mL). After stirring for 24 h, the solution was filtered through a medium pore size sintered glass to remove insoluble substances. The chitosan solution was then cast into film on a Teflon-coated mold (8x8 inch in size). The solvent was allowed to evaporate in air for 4-5 days. The chitosan film was peeled off and immersed in 0.1 M NaOH/methanol (1:1) and methanol/water (1:1) to neutralize the acid used as a solvent. The film was dried under vacuum for more than 1 day. The film thickness was between 40 to 100 μm.

## 2.4 Synthesis of MTEG-ald

To the solution of oxalyl chloride (34.37 mmol) in DCM (15 mL) under $N_2$ and cooled in a dry-ice-acetone bath. DMSO (70.57 mmol) in 5 mL of DCM were carefully added. The solution was stirred for 5 min, and then a solution of MTEG (29.7 mmol) in 10 mL of DCM was added dropwise. The mixture was stirred for 3 h, and then TEA (143.76 mmol) was added dropwise over a period of 20 min. The reaction mixture was left for 30 min at –78 °C and then allowed to reach room temperature. The crude products were concentrated by rotary evaporator. $^1$H NMR δ 3.1 (3H, s, $OCH_3$), 3.3-3.4 (8H, t, $OCH_2CH_2$), 3.9 (2H, s, $C\underline{H}_2CHO$), 9.6 (1H, s, CHO).

## 2.5 Synthesis of MPEG–ald

Oxalyl chloride (19.2 mmol) was dissolved in DCM 15 mL under $N_2$, the solution was cooled to –78 °C, DMSO (40.8 mmol) in DCM 5 mL was slowly added over a period of 3 min. Dried MPEG (4.8 mmol) in DCM 10 mL was added using

addition funnel. The mixture was stirred at –78 °C for 5 h, treated with TEA (71.7 nmol), and allowed to warm to ambient temperature. The crude products were concentrated by rotary evaporator. $^1$H NMR δ 2.9 (3H, s, $OCH_3$), 3.1-3.3 (40H, m, $OCH_2CH_2$), 3.8 (2H, s, $C\underline{H}_2CHO$), 9.6 (1H, s, CHO).

## 2.6 Reaction between chitosan and butyraldehyde (homogeneous state)

Chitosan (1 equiv of $NH_2$) was dissolved in 20 mL of 2 % acetic acid. The solution of butyraldehyde (1 equiv) in MeOH (80 mL) was added to the diluted chitosan solution and stirred at room temperature. The mixture turned to gel or suspension after 1-6 min. $NaCNBH_3$ (1.2 equiv) was added and stirred for 1 day. Yellowish precipitate was formed and filtered out. It was used to wash the product which was later dried in vacuum.

## 2.7 Reaction between chitosan and MTEG-ald (homogeneous state)

Chitosan (1 equiv. of $NH_2$) was dissolved in a mixture of aqueous 2 % acetic acid solution (20 mL) and methanol (5 mL). MTEG-ald (10 equiv) was added to the above chitosan solution and stirred for 30 min at room temperature. Then the pH of chitosan/ MTEG-ald solution was adjusted to 6.5 with aqueous 1 M NaOH solution and stirred for 60 min at room temperature. At this point, no precipitate was observed. $NaCNBH_3$ (10 equiv) in 5 mL water was added to the reaction mixture dropwise over a period of 20 min. The solution was stirred for 3 days at room temperature. The reaction mixture was dialyzed with dialysis membrane (Mw cut-off = 12,000) against deionized water. The solution was evaporated and washed twice with 100 mL acetone, then dried in vacuo.

## 2.8 Reaction between chitosan and MPEG-ald (homogeneous state)

The procedure of this reaction is the same as reaction between chitosan with MPEG-ald, except MPEG-ald was used instead of MTEG-ald.

## 2.9 Reaction between chitosan films and butyraldehyde

An aqueous solution of butyraldehyde (-CHO 10 equiv) was dissolved in methanol 20 mL and was added into a flask containing chitosan films (1 equiv). The mixture was stirred for 30 min at room temperature. $NaCNBH_3$ (10 equiv) in 5 mL methanol was added to the reaction mixture dropwise for 20 min and the solution was stirred for three days at room temperature. Chitosan films were later washed in methanol then dried in vacuo. Stoichiometric ratios of chitosan: aldehyde: $NaCNBH_3$ were 1:10:10 and 1:30:30 that were solved in MeOH or DMF.

## 2.10 Reaction between chitosan films and MTEG-ald

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## 2.12 Contact angle measurements

Contact angle meter model CAM-PLUS MICRO was used for the determination of water contact angles. A droplet of 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 is projected on the screen and the angle is measured. All measurements were performed at 22-25 $^{o}$C.

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