CHAPTER II

EXPERIMENTAL

2.1 Experimental design

To address the first hypothesis, charged functional groups to be introduced to the amino groups of chitosan are quaternary ammonium and sulfonated group. The optimized condition and the ability to control the extent of surface modification is investigated by varying some experimental parameters including temperature and time. All modified chitosan films were analyzed for surface functionality and atomic composition by ATR-IR and XPS, respectively. Finally, charged characteristic on both surfaces was evaluated by zeta-potential measurement.

To address the second hypothesis, six batches of positively- and negatively-charged chitosan films are tested quantitatively for protein adsorption. Four proteins having distinctive charge and size were used for the test.

2.2 Materials

Chitosan (M_V = 645,535) Da was obtained from Seafresh Chitosan (Lab) Co., Ltd. (DAC-88). Methanol was distilled over 4A molecular sieves. Methyl iodide, 5-formyl-2-furan-sulfonic acid, sodium borohydride, sodium hydroxide and sodium iodide were purchased from Fluka Chemika and used as received. Bovine serum albumin, fibrinogen, lysozyme, ribonuclease, bicinchoninic assay kit and phosphate buffer saline (PBS) were purchased from Aldrich Chemical Co.

2.3 Equipment

2.3.1 Nuclear magnetic resonance (NMR) spectroscopy

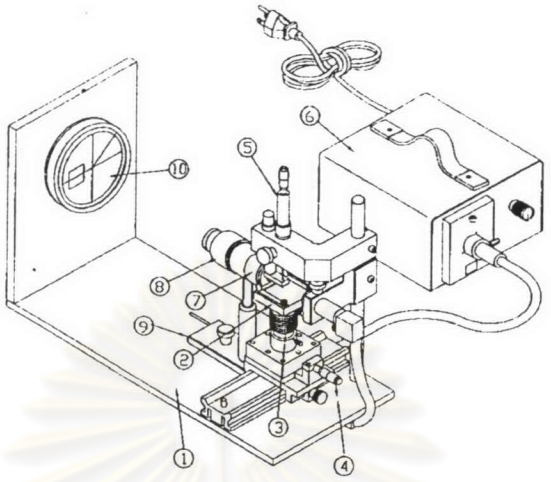
NMR spectra were obtained from 1% solution in 1% CF_3COOH D_2O or D_2O using 400 MHz (^1H) on Varian mercury-400 spectrometer. Chemical shifts are reported in ppm.

2.3.2 Attenuated total reflectance infrared (ATR-IR) spectroscopy

All spectra were collected at resolution of 4 cm^{-1} and 128 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.

2.3.3 Air–water contact angle measurement

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 °C.



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

2.3.4 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectra were obtained on a thermoVGscientific using Mg K_α excitation. In this study, the take off angle at 45° was chosen and the approximate of depth profile is 30 Å.

2.3.5 Zeta-potential measurement

Zeta-potential model ESL 8000 (otsuka Electro, Co, Tokyo, Japan) was used for measure charge characteristic on sample surface. Pieces of films were cut to fit the recesses of the modified rectangular cell and immersed in distilled water and stored at room temperature for 12 h to equilibrate the surface. The zeta-potential of sample was then measured at 10 mM.

2.3.6 UV-spectroscopy

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

2.4 Preparation of chitosan films

Chitosan (0.8 g) was dissolved in 0.1 M acetic acid (40 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 petri dish (2.5 inch in diameter 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 60 to 100 μm.

2.5 Preparation of positively-charged chitosan film

Anhydrous MeOH (7.0 ml) was added into a flask containing chitosan films (1 equiv of NH_2) and NaOH (3 equiv). NaI was added to adjust the the concentration in the reaction medium to 0.2 mol/L. Subsequently, methyl iodide was added to the mixture with 2 steps (3 equiv in each step) and the reaction was carried out at 40°C for 8 h. Then the films were rinsed twice with MeOH, and dried under vacuum for more than 3 days. Stoichiometric ratios of chitosan: MeI were 1:3, 1:6 and 1:12.

2.6 Preparation of negatively-charged chitosan film

FFSA (1 equiv) was dissolved in 10 mL methanol and was added into a flask containing chitosan films (1 equiv of NH_2). The mixture was stirred for 20 min at room temperature. $NaBH_4$ (1 equiv) was added into the reaction mixture and the solution was stirred for 24 h at room temperature. Chitosan films were later washed in methanol then dried under vacuum. Stoichiometric ratios of chitosan: FFSA: $NaBH_4$ were 1:1:1, 1:3:3, 1:5:5 and 1:12:12.

2.7 Protein adsorption

Protein solutions were freshly prepared by dissolving BSA, FIB, lysozyme and RNase in PBS at pH 7.4 to give a final concentration of 1 mg/mL. To reach an equilibrium hydration, the film substrate was immersed in the PBS solution overnight prior to adsorption. Each sample was removed from PBS solution and suspended into the wells containing 3 mL protein solution before incubated at 37 °C for 3 h. Then the films were removed and rinsed with 4x10 mL PBS solution to remove reversibly adsorbed protein. To remove irreversibly adsorbed protein from the film surface, each film was transferred to another vial containing 1.0 mL of 1.0 wt% sodium dodecylsulfate (SDS), soaked for 1h at room temperature. To determine the total amount of protein adsorbed on the substrates, micro-bicinchoninic acid (BCA) protein assay was utilized [29]. The 0.1 mL solution was withdrawn from the vial and mixed with 0.1 mL BCA working solution in a well-plate. The mixed solution was left stand at room temperature for 16 h to maximize color development. The absorbance of the solution was measured at 562 nm by UV-VIS spectroscopy (96 well Microtiter plates reader). The amount of adsorbed protein was determined by comparison with the absorbance of the samples to a calibration curve. Three repetitions were performed for all samples.