CHAPTER III

EXPERIMENTAL

3.1 Materials

1. Acetaldehyde : Fluka
2. Acetic acid : Carlo
3. Benzaldehyde : Merck
4. Butyl iodide (BuI) : Fluka
5. Butyraldehyde : Sigma
6. Chitosan (MW=550,000) : Seafresh Chitosan (Lab) Co.,Ltd.
7. *Escherichia coli (E. coli)* : National Center for Genetic Engineering and Biotechnology (Biotec)
8. Ethanol : Merck
9. Ethyl iodide (EtI) : Sigma
10. Formaldehyde : Merck
11. Glutaraldehyde : Fluka
12. Methanol : Merck
13. Methyl iodide (MeI) : Fluka
14. Octyl iodide (OcI) : Fluka
15. Phosphate buffer saline (PBS) : Sigma
16. Propionaldehyde : Fluka

17. Sodium borohydride ($NaBH_4$) : Fluka
18. Sodium hydroxide : Fluka
19. Sodium iodide : Fluka
20. *Staphylococcus aureus (S. aureus)* : National Center for Genetic Engineering and Biotechnology (Biotec)
21. Triplicate soy agar (TSA) : PPL system Co., Ltd.
22. Triplicate soy broth (TSB) : PPL system Co., Ltd.
23. Tripolyphosphate : Fluka

3.2 Equipment

3.2.1 Contact Angle Measurements

A contact angle goniometer equipped with a Gilmont syringe and a 24-gauge flat-tipped needle (Ramé-Hart, Inc., U.S.A.) was used for the determination of water contact angles. A droplet of Milli-Q water was placed on the tested surface by bringing the surface into contact with a droplet suspended from a needle of the syringe. The angle was promptly measured within 5 seconds after water drop arrived at the film surface. The rapid measurement was necessary to assure that there is no error caused by water absorption of the film surface. The measurements were carried out in air at the room temperature. The reported angle was an average of 5 measurements on different area of each sample.

3.2.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H NMR spectra were recorded in 1% solution of CF_3COOH/D_2O or D_2O using a Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts were reported in part per million (ppm)

relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.3 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

All spectra were collected at a resolution of 4 cm^{-1} and 128 scans using a Nicolet Magna 750 FT-IR spectrometer equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector. A single attenuated total reflection accessory with 45° germanium (Ge) IRE (Spectra Tech, U.S.A.) and a variable angle reflection accessory (Seagull™, Harrick Scientific, U.S.A.) with a hemispherial Ge IRE were employed for all ATR spectral acquisitions.

3.2.4 UV-Spectrometer and Microplate Reader

UV spectroscopy Model MV, the Bausch ε Lomb and Multi-Detection Microplate Reader Model All, Bio-Tek Instruments, Inc., U.S.A. was used for determination of the optical density of bacteria suspension.

3.2.5 Scanning Electron Microscopy

Initial adhesion of bacteria on modified chitosan films was examined under a scanning electron microscope (JEOL Model JSM-5800L, Japan).

3.2.6 Zeta-potential Measurement

The zeta-potential of chitosan particles before and after surface modification was determined using a Zetasizer Nano-ZS (Malvern Instruments, UK). The particles were dispersed in Milli-O water. The analysis was performed at 25°C using a scattering angle of 173°.

3.3 Methods

3.3.1 Preparation of Chitosan Films

Chitosan flakes (2 g) were dissolved in 0.1 M aqueous acetic acid solution (100 mL). After stirring for 24 h, the solution was filtered through a sintered glass having a medium pore size to remove insoluble substances. The chitosan solution was then cast into a film on a Petri dish (2.5 inches in diameter). The solvent was allowed to evaporate in air for 4-5 days. The chitosan film was peeled off from the dish and immersed in 1:1 (v/v) 0.1 M NaOH/methanol and 1:1 (v/v) methanol/water to neutralize the acid used as a solvent. The film was dried under vacuum for 1-2 days. The film thickness was 60-100 μm. During the neutralization-drying steps (in NaOH-MeOH solution), the chitosan films swelled up and shrank, resulting in scattered wrinkle area across the film. Only selected smooth areas of the films were, however, used for surface modification.

3.3.2 Preparation of Chitosan Particles

Chitosan was dissolved at 0.5 % (w/v) with 1% (v/v) acetic acid. pH of the solution was raised to 4.6-4.8 with 10 N NaOH. Chitosan particles formed spontaneously upon an addition of 1 mL of an aqueous tripolyphosphate solution (0.25%, w/v) to 3 mL of chitosan solution under magnetic stirring. The particles were isolated by centrifugation at 6000 rpm for a 30 min interval. Supernatants were discarded and the particles were extensively rinsed with distilled water and then freeze-dried before further use or analysis.

3.3.3 Preparation of *N*-alkyl Chitosan Films or Particles

An anhydrous methanol solution of a selected aldehyde (10 mL) having a desired concentration (0.4 -1 M) was added into a flask containing chitosan films (2×2 cm^2) or particles (0.03 g). After stirring for a given time at room temperature, $NaBH_4$ (0.3 g, 0.8 mol) was added into the reaction mixture and the solution was stirred for 24 h at room temperature. The films were removed from the solution, rinsed thoroughly with methanol, and dried under vacuum. In the case of particles, they were

isolated by centrifugation at 6000 rpm for 30 min. Supernatants were discarded and the particles were extensively rinsed with methanol and dried under vacuum.

3.3.4 Preparation of Quaternized *N*-alkyl Chitosan Films or Particles

An anhydrous methanol solution of NaI (0.2 M) was added into a flask containing N-alkyl chitosan films (2×2 cm^2) or particles (0.03 g) and NaOH (0.13 g, 0.3 mol). A selected alkyl iodide was added via syringe. The total volume of the reaction mixture was 10 mL and the concentration of alkyl iodide was varied in the range of 0.4 – 2.4 M. The reaction mixture was stirred at 50°C for a given time. The films were removed from the solution, rinsed thoroughly with methanol, and dried under vacuum. In the case of particles, they were isolated by centrifugation at 6000g for 30 min. Supernatants were discarded and the particles were extensively rinsed with methanol and dried under vacuum.

3.3.5 Evaluation of Antibacterial Activity

Triplicate soy broth (TSB) was used as a growth medium for the antibacterial assays, *Staphylococcus aureus (S. aureus)* and *Escherichia coli* (*E. coli*) were used as gram positive and gram negative bacteria, respectively. All glassware used for the tests was sterilized in an autoclave at 121°C for 15 min prior to use. The quaternized *N*-alkyl chitosan films were sterilized by exposing to UV radiation for 30 min prior to the tests.

3.3.5.1 Shake Flask Method

A 0.3 mL bacterial suspension in distilled water was pipetted into a flask containing 50 mL TSB. Quaternized *N*-alkyl chitosan films (2×2 cm^2) were immersed in the bacterial solution and shaken at 250 rpm at 37°C. Three replicates were used for each sample. After 24 h, the films were removed from the solution by a sterile forceps and immersed in 3% glutaraldehyde solution at 4°C in order to fix the bacteria that adhered on the films. After 24 h, the glutaraldehyde solution was removed and the films were washed with PBS, followed by a stepwise dehydration with 30%, 50%,

70%, 90% and 100% ethanol in water for 10 min interval. The films were then dried and sputter-coated with a thin film of gold before being characterized by scanning electron microscopy. The optical density of the solution was measured at 600 nm (OD_{600}) by UV-Vis spectroscopy to determine a density of bacteria in the broth. The dry weight of bacteria in the broth (g/L) was determined by comparing the optical density at 600 nm (OD_{600}) of the sample with a calibration curve.

3.3.5.2 Plate Counting Method

A quaternized *N*-alkyl chitosan film (1×1 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 was pipetted into the well. The well plate was incubated in an incubator shaker at 37°C, 110 rpm for 24 h. Then, the bacterial solution (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 solution was diluted to 10^{10} times. A 100 μL of the diluted bacterial solution was then spreaded onto the triplicate soy agar. 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). The antibacterial ratio was calculated using the following relationship;

$$\text{Antibacterial ratio} = \frac{\text{Number of original cell - Number of viable cell}}{\text{Number of original cell}} \times 100\% \quad \text{....(3.1)}$$