CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials Used in the Preparation of *N*-trimethyl Chitosan Chloride

- Chitosan (200,000 g mol^{-1}; Seafresh, Thailand)
- Methyl iodide (CH_3I; Merck, Germany)
- Sodium chloride (NaCl; Univar, USA)
- Sodium hydroxide (NaOH; Aldrich, USA)
- Sodium iodide (NaI; Aldrich, USA)
- Ethanol (Lab-Scan (Asia), Thailand)
- N-methyl-2-pyrrolidone (NMP; Lab-Scan (Asia), Thailand)

3.1.2 Materials Used in the Preparation of Carboxymethyl Chitosan

- Chitosan (200,000 g mol^{-1}; Seafresh, Thailand)
- Monochloroacetic acid ($ClCH_3COOH$; Acros Organics, Belgium)
- Sodium hydroxide (NaOH; Aldrich, USA)
- Isopropyl alcohol (Honeywell Burdick & Jackson, Korea)

3.1.3 Material Used in the Fabrication of Blended Hydrogel

- Poly(vinyl alcohol) (PVA; 85,000-124,000 $gmol^{-1}$, Aldrich, USA)

3.1.4 Materials Used for Antibacterial Activity Assay

- Luria-Bertani Broth (LB Broth; Difco Becton Dickinson., USA)
- Agar Count plate (Difco Becton Dickinson and Company.,USA)
- Luria-Bertani Agar (LB Agar; Difco Becton Dickinson., USA)
- Muller-Hinton Broth (MU Broth; Difco Becton Dickinson., USA)
- Muller-Hinton Agar (MU Broth; Difco Becton Dickinson., USA)
- Sodium chloride (NaCl; Univar)

3.1.5 Materials Used for Cell Culture

- Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA)

- 10% Fetal bovine serum (FBS; BIOCHROM AG)
- 1% L-glutamine (Invitrogen Corp., USA)
- 1% Antibiotic (Invitrogen Corp., USA)
- 1% Lactabomine (Invitrogen Corp., USA)

3.2 Equipments

3.2.1 Fourier Transform Infrared spectrometer (FTIR; Nicolet, Nexus 670)
3.2.2 Freeze Dry (LABCONCO)
3.2.3 Nuclear Magnetic Resonance Spectrometer (NMR; Varian INOVA)
3.2.4 Scanning Electron Microscope (SEM; A JEOL JSM 5410LV)
3.2.5 UV-Visible Spectrophotometer

3.3 Methodology

3.3.1 *N*-trimethyl Chitosan Chloride (TMC) Preparation

TMC was synthesized by the modified method of Chang *et al.*, (2009). TMC was synthesized via quaternization by using CH_3I. A mixture of 2 g of chitosan, 4.8 g of NaI , 11 ml of 15 %w/v aqueous NaOH solution, and 11.5 ml of CH_3I were added into 80 ml of NMP in a round bottom flask, then the mixture was stirred in a oil bath at 60°C for 90 min, the mixture changed to yellow transparent solution. The product was precipitated by adding 200 ml of ethanol, and then isolated by centrifugation at 10,000 rpm for 10 min. After washing with 100 ml of ethanol and centrifugation, the product was dissolved in 40 ml of 10 %w/v aqueous NaCl solution to exchange the iodide ions with chloride ions. The product was purified by dialysis against distilled water for 5 days. Final step, the product was freezed at -45°C and lyophilized at -50°C. The product was kept in dessicator until used.

3.3.2 Carboxymethyl Chitosan (CM-chitosan) Preparation

CM-chitosan was synthesized via methylation by using $ClCH_2COOH$ bases on the method discribed by Chen *et al.*, (2003). A mixture of 10 g of chitosan and 13.5 g of NaOH were added into a solution in a round bottom flask, that solution

consists of 20 ml of distilled water and 80 ml of isopropanol. The mixture was maintained in a ice bath at low temperature for 1 h to swell and alkalize. An additional $ClCH_2COOH$ solution was added into the reaction mixture by dropwise for 30 min and reacted for 4 h at the same temperature that solution was prepared by dissolving 15 g of $ClCH_2COOH$ into 20 ml of isopropanol. The reaction was stopped by adding 200 ml of 70% ethanol into the reaction. The solid was filtered and rinsed in 70–90% ethanol to desalt and dewater, and vacuum dried at room temperature. The products were Na salt form of CM-chitosans.

1 g of Na salt CM-chitosan was suspended in the mixture of 100 ml of 80% ethanol and 10 ml of 37% of hydrochloric acid for 30 min. The solid was filtered and rinsed in 70–90% ethanol to neutral, and vacuum dried at room temperature. The final products were the H-form of CM-chitosans.

3.3.4 Blended Hydrogels Preparation

Preparation of the blend hydrogels is followed and adapted from Zhao *et al.*, (2007). First step is the preparation of polymer solution, PVA was dissolve in distilled water at 80°C for 3 h and CM-chitosan was dissolve in distilled water at room temperature and then added CM-chitosan into PVA solution after that stirred the mixture to form homogeneous solution at 60-70°C for 30 min. PVA in the final solution is 10 %w/v and the content of CM-chitosan in the solution varied from 5 to 20 %w/w of PVA content.

Second step is an additional of TMC into the blended solution at the same temperature by varying the content of TMC from 5 to 15 %w/w of PVA content. The blended solution was packed in the nylon bags. The blended solution was irradiated with 25-45 kGy of the ^{60}Co gamma rays at room temperature.

3.4 Characterization

3.4.1 Fourier-Transformed Infrared Spectrophotometer (FT-IR)

FTIR spectroscopy was used to investigate chemical functionalities of TMC and CM-chitosan. Infrared spectra with a resolution of 4 cm^{-1} of the samples as KBr pellets were recorded by Fourier Transform Infrared spectrometer.

3.4.2 Nuclear Magnetic Resonance Spectrometer (NMR)

The functional groups and structures of TMC and CM-chitosan were investigated by using Nuclear Magnetic Resonance Spectrometer and used to determine the degree substitution of TMC and CM-chitosan.

3.4.3 Scanning Electron Microscope (SEM)

The morphology of the blend hydrogels was studied by Scanning Electron Microscope. The blended hydrogels were placed on brass stubs and then they were coated with gold, and observed for their microscopic morphology by using scanning electron microscopy.

3.4.4 UV-Visible Spectrophotometer

UV/Vis spectrophotometry was used to determine the turbidity of the mediums that mediums were used to culture a microbial and to detect the cells in cytotoxicity test.

3.5 Physical Properties of the Blended Hydrogels

3.5.1 Gel Fraction of the Blended Hydrogels

The gel fraction of the blended hydrogels was investigated by using follow method, the sol was extracted in distilled water at 121 °C for 4 h in an autoclave (Zhai *et al.*, 2002) The remained gel was dried to constant weight at 60 °C.

The gel fraction is define as:

$$\%Gel\,fraction = \frac{W_g}{W_o}\ x\ 100, \qquad (1)$$

Where W_g was weight of dry hydrogel after extraction.

W_0 was the initial weight of dry hydrogel.

3.5.2 Swelling of the Blended Hydrogels

Swelling behavior of the blended hydrogels was investigated by immersing the blended hydrogels into the simulated body fluid pH 7.4 (SBF) at 37 °C for different time. The excessive surface water was removed with filler paper. The degree of swelling of the blended hydrogels was calculated according to the following equation:

$$\%\, Swelling = \frac{W_s - W_i}{W_i}\ x\ 100\ , \quad (2)$$

Where W_s was weight of swollen hydrogel.

W_i was the initial weight of dry hydrogel.

3.5.3 Weight Loss of the Blend Hydrogels

The weight loss of the blended hydrogels was studies by immersing the dry hydrogels into the simulated body fluid pH 7.4 (SBF) at 37 °C for different time. The remained gel was dried to constant weight at 60 °C. The weight loss percent was calculated according to the following equation:

$$\%\, Weight\ loss = \frac{W_i - W_1}{W_i}\ x\ 100\ , \quad (3)$$

Where W_i was the initial weight of dry hydrogels.

W_1 was the weight of dry hydrogels after immersed in SBF.

3.5.4 Water Absorption of the Blended Hydrogels

The water absorption of the blended hydrogels was studies by immersing the dry hydrogels into the distilled water at room temperature to reach a constant weight. Before weighing the sample, any surface water was removed with filter paper. The water absorption percent was calculated according to the following equation:

$$\%\, Water\ absorption = \frac{W_s - W_i}{W_i}\ x\ 100, \quad (4)$$

Where W_i was the initial weight of dry hydrogels.

W_s was the weight of swollen hydrogels.

3.5.5 Molecular Weight Between Crosslink of the Blended Hydrogels

The molecular weight between crosslink (Mc) of the blended hydrogels was studied. Mc was determined by using the equilibrium swelling data and Flory and Rehner equation (Bajpai *et al.*, 2005, Dhara *et al.*, 1999 and Gudeman *et al.*, 1995):

$$M_c = \frac{-V_1 d_p(v_s^{\frac{1}{3}} - v_{s/2})}{\ln(1-v_s)+v_s+\chi v_s^2}, \qquad (5)$$

$$v_s = \frac{1}{swelling\ ratio}, \qquad (6)$$

$$V_e = d_p \frac{N_A}{M_c}, \qquad (7)$$

Where V_1 was the molar volume of water (ml/mol).

d_p was the polymer density (g/ml).

χ was the Flory-Huggins constant.

v_s was the polymer volume fraction in the equilibrium swollen polymer.

V_e was the crosslink density

N_A was the Avogadro's number ($6.022 \times 10^{23} mol^{-1}$)

3.5.6 Moisture Retention Capability of the Blended Hydrogels

The moisture retention capability of the blended hydrogels was measured by the water losing rate and the ratio of water holding in the hydrogel for different time. The sample with thickness of 3 mm, radius of 13 mm, and weight of about 0.6 g, was put into a petri dish at room temperature. Moisture retention capability was calculated as follows:

$$\% \, Moisture \, retention \, capability(Rh) = \frac{M_t}{M_i} x \, 100, \quad (8)$$

Where M_i was the initial weight of hydrogels.

M_t was the weight of hydrogels at different time.

3.5.7 Water Vapor Transmission Rate of the Blend Hydrogels

The water vapor transmission rate (WVRT) of the blended hydrogels was measured by measuring the weight loss of a bottle which contains 3 ml of water. The bottle has a mouth with a diameter of 13 mm. The sample with a diameter of 25 mm was put on the mouth of the bottle as a cap, then placed in an oven at 35°C for 24 h. The water vapor transmission rate (WVRT) was determined by using the following equation:

$$WVRT = \frac{W_i - W_t}{A \, x \, 24} \, x \, 10^6 \, gm^2/h \quad , \quad (9)$$

Where W_i was the weight of bottle before placed in oven.

W_t was the weight of bottle after placed in oven.

A was the area of bottle mouth (mm^2).

3.6 Antibacterial Activity Assay

3.6.1 Minimum Inhibitory Concentration (MIC) of TMC and CM-chitosan

Dilution methods are used to determine the minimal concentration of inhibition to bacteria. The minimum inhibition concentration (MIC) values were studied for antibacterial activity of *Acinetobacter lwoffii* ATCC 15309, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* DMST 20654 (MRSA), *Staphylococcus epidermidis* ATCC 12228, and *Listeria monocytogenes* DMST 17303 by agar dilution (Barry *et al.*, 1976). First step, TMC and CM-chitosan stock solution (60 mg/ml in distilled water) were two-fold serial diluted by using 2 ml of the sterile Muller-Hinton Broth (MU broth) to different concentrations and were added the sterile melted Muller-Hinton Agar (MU agar)

with the final volume 5 ml. The mixed solutions were immediately pour into petri dishes (50 mm in diameter) after vortexing.

Second step was the preparation of microbial culture, in this step the bacterial were cultured into 2 ml of Luria-Bertani Broth (LB broth) for overnight. The bacterial culture should be diluted to contain 10^5 to 10^6 cells/ml. The bacterial culture was transferred to 2 ml of 0.85%w/v of aqueous NaCl solution lead to the turbidity of 0.85%w/v of aqueous NaCl solution equal to McFarland standard No. 0.5 (10^8 cells/ml) after that added 50 µl of microbial in the aqueous NaCl solution to 2 ml of MU broth and mixed together.

Final step, the petri dishes in first step that were spot inoculated with 10 µl (10^4 cells) of each bacterial strains and incubated at 37°C overnight. After 24 h of incubation, MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as standard reference strains.

3.6.2 Colony Count Method

Antibacterial activity of the blended hydrogels was investigated by using the colony count method. The test was conducted against Gram-nagative *Acinetobacter lwoffii* ATCC 15309 and *Escherichia coli* ATCC 25922 and Gram-positive *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* DMST 20654 (MRSA), *Staphylococcus epidermidis* ATCC 12228, and *Listeria monocytogenes* DMST 17303. First step was the preparation of bacterial culture, the bacteria were cultured into 2 ml of LB broth at 37 °C for overnight. The bacterial culture was diluted to contain 10^5 to 10^6 cells/ml. The bacterial culture was transferred to 2 ml of 0.85 %w/v of NaCl solution lead to the turbidity of 0.85%w/v of NaCl solution equal to McFarland standard No. 0.5 (10^8 cells/ml).

Second step, the blended hydrogels (0.2 g) was added into the LB broth (2 mL), and transferred 10^4 cells in 0.85%w/v of NaCl solution (20 µL) into LB broth that contains the blended hydrogels, then the mixtures were shaken with 240 rpm at 37°C for 24 h.

Final step, 100 µL of mixtures was dipped and spread on agar plate count in Petri dishes (90 mm) and incubated at 37 °C for 24 h. The bacterial

reduction rate (BRR) for each type of hydrogels against a microbe was calculated by using the following equation:

$$BRR = \frac{N_1 - N_2}{N_1} x\, 100\,, \tag{10}$$

Where N_1 was the number of colonies of the bacterial suspension without the blended hydrogels.

N_2 was the number of colonies of each sample suspensions.

3.7 Cytotoxicity of the Blended Hydrogels

3.7.1 Indirect Cytotoxic Assay

The toxicity of blended hydrogels was studied via an indirect cytotoxic test by using mouse fibroblast cells (L929). First, extraction media was prepared by immersing 20 mg of the blended hydrogels in 2 ml of a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1 % lactabumin, and 1% antibiotic and antimycotic) for 1 and 7 d. Each of these extraction media was used to evaluate the cytotoxicity of the blended hydrogels. Second, L929 was separately cultured in wells of a 24-well culture plate in 10% serum-containing DMEM for 16 h to allow cell attachment on the plate. Then, the cells were starved with SFM for 24 h, after which time the medium were replaced with an extraction medium. After 24 h of cell culturing in the extraction medium, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out to quantify the amount of viable cells.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each culture medium was aspirated and replaced with 300 μl/well of MTT solution at 5 mg/ml without phenol red and the plate was incubated for 10 min at 37°C. The solution was aspired and 900 μl/well of dimethylsulfoxide (DMSO) containing 125 μl/well of glycine buffer (pH=10) was added to dissolve the formazan crystals. Finally, after 10 min of rotary

agitation, the absorbance of the solution was measured at 570 nm by using UV/Visible spectrophotometer, that absorbance represents the amount of viable cells.