CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Chitin Flakes

Chitin flakes from shrimp shells were purchased from Seafresh Chitosan (Lab) Company Limited, Thailand.

3.1.2 Chemicals

2-acrylamido-2-methylpropane sulfonic acid (AMPS) was purchased from Merck Chemicals, Germany. *N-N'*-methylenebisacrylamide (MBA) and 1-Hydroxycyclohexyl phenyl ketone were purchased from Sigma-Aldrich.

3.1.3 Solvents

Anhydrous NaOH pellets and hydrochloric acid (HCl) (37% w/w) were of analytical grade and purchased from Carlo Erba (Italy). Hydrogen peroxide 100 (volumes > 30% w/v) was purchased from Fisher Scientific (NJ, USA). Phosphate-buffered saline (PBS) was purchased from Mediatech, Inc. (Manassas, VA). All of solvents were used without further purification.

3.1.4 Enzyme

Lysozyme from hen egg white was purchased from Fluka.

3.2 Equipments

3.2.1 Fourier-Transformed Infrared Spectrophotometer (FT-IR)

3.2.2 Transmission Electron Microscope (TEM)

3.2.3 Scanning Electron Microscope (SEM)

3.3 Methodology

3.3.1 Preparation of Chitin Whiskers

Chitin flakes have been soaked in hydrogen peroxide mixed with 37% hydrochloric acid in 9:1 solution overnight for bleaching. Chitin whiskers were

prepared as previously described by Paillet and Dufresne with some modifications. In brief, the whisker suspension was obtained by hydrolyzing chitin sample with 3N HCl at 104°C for 6 h under vigorous stirring. The ratio of the 3N HCl solution to chitin was 30 cm^3 g^{-1}. After acid hydrolysis, the suspension was immediately bleached again with hydrogen peroxide: hydrochloric acid (9:1) solution, and then diluted with distilled water, followed by centrifugation to separate the chitin whisker solid residues from the aqueous medium, because of the nanocrystalline nature of the whiskers, the centrifugation could be achieved at 10,000 rpm for 10 min. This process was repeated three times. The suspension were then transferred to a dialysis bag and dialyzed in distilled water at room temperature to remove HCl that could be remained in the suspension until pH of distilled water is about 6. Finally, the suspension was kept in a refrigerator prior to further use.

3.3.2 Synthesis of Hydrogels

3.3.2.1 Preparation of 2-Acrylamido-2-Methylpropane Sulfonic Acid Sodium Salt (AMPS-Na^+) Solution

2-acrylamido-2-methylpropane sulfonic acid sodium salt (AMPS-Na^+) solution polymerization was carried out in distilled water as the solvent at room temperature using various monomer concentrations ranging from 30-50% (w/v). In the case of AMPS-Na^+ at concentration of 30% (w/v), the monomer solution was was prepared by first dissolving 30 g of 2-acrylamido-2-methylpropane sulfonic acid (AMPS) in about 30 ml of distilled water and adding to this solution 25 ml of a 30% (w/v) NaOH solution under cooling. Then, the solution was titrated with 1 M NaOH to pH 7.00. Finally, the volume of the solution was adjusted to 100 ml with distilled water. The 40% (w/v) and 50% (w/v) AMPS-Na^+ solutions were prepared using the same procedure using 40 g and 50 g AMPS, respectively.

3.3.2.2 Preparation of 2-Acrylamido-2-Methylpropane Sulfonic Acid Sodium Salt (AMPS-Na^+) Hydrogels by Ultraviolet Radiation and Gamma Radiation

For polymerization, *N-N'*-methylenebisacrylamide (MBA) were added to monomer solution as crosslinking agent in concentrations ranging from 0.1-1% (mol/mol monomer) and 0.01 % (w/w) of 1-Hydroxycyclohexyl phenyl

ketone were employed as photoinitiator. 5 ml of the total mixture was transferred to nylon bag in the size of 5×5 cm^2 and sealed nylon bag with heat. Finally, all the formulations were irradiated under UV lamp in the wavelange range between 280-390 nm with maximum intensity of 18.16 mW/cm^2 in the distance of 10 cm from the lamp for 5 min to form into hydrogel sheet. In the case of Gamma radiation, the monomer solution was only added with *N-N'*-methylenebisacrylamide (MBA) in concentrations ranging from 0.1-1% (mol/mol monomer) and also 5 ml of total mixture was transferred to 5×5 cm^2 of nylon bag and sealed, after that all the formulations were irradiated under Gamma radiation at radiation dose of 25 kGy to form hydrogel.

3.3.2.3 Preparation of Chitin Whiskers Reinforced AMPS-Na⁺ Hydrogels by Ultraviolet Radiation and Gamma Radiation

The as-prepared chitin whisker suspensions in varying amount were then added to the AMPS-Na^+ solution (with appropriate addition of distilled water to obtained 100 ml of the final suspension) to achieve the whisker content in the range of 3-8 wt%. The AMPS-Na^+/chitin whisker suspension was added with required amount of *N-N'*-methylenebisacrylamide (MBA) crosslinker and 1-Hydroxycycloxehyl phenyl ketone photoinitiator (only for Ultraviolet radiation technique). The total mixture was further stirred mechanically before transferred to nylon bag and irradiated under Ultraviolet radiation and Gamma radiation.

3.4 Characterization

3.4.1 Characterization of Chitin Whiskers

3.4.1.1 Fourier Transformed Infrared Spectroscope (FTIR)

Chemical integrity of the chitin whiskers was investigated by a Fourier-transformed infrared spectroscope (FTIR) (Thermo Nicolet, Nexus 670) with 64 scans at a resolution of 4 cm^{-1} in a frequency range of 4000-400 cm^{-1}. The chitin whiskers from the as-prepared chitin whisker suspension were dried, mixed with KBr powder, and pressed into a pellet.

3.4.1.2 Transmission Electron Microscope (TEM)

Morphological appearance and sizes of the as-prepared chitin whiskers were observed using a JEOL JEM-2100 Transmission Electron Microscope (TEM). Samples of chitin whiskers were prepared from a drop of a dilute chitin whiskers suspension which was deposited and left to dry on a formvar grids before TEM observations. The average dimensions of the whiskers were determined from the selected TEM images, from which at least 200 whiskers were measured for their length and width using SemAfore 4.0 image-analytical software.

3.4.2 Characterization of Hydrogels

3.4.2.1 Determination of Gel fraction

The samples were dried after irradiation (W_0), then extracted in distilled water at 121°C for 4 h in an autoclave in order to remove the soluble parts. The gels were then dried again in vacuum oven to a constant weight (W_E). The gelation% was calculated by the following equation (1):

$$\text{Gelation } (\%) = \frac{W_E}{W_0} \times 100 \qquad (1)$$

Where W_E is the dried weight of the sample after extraction of soluble parts.

W_0 is the weight of dried gel after irradiation.

3.4.2.2 Measurement of Swelling Capacity

In order to check the swelling behavior of hydrogels for using as wound dressings, Simulated body fluid (SBF) was used to investigate the swelling behavior of hydrogels which are directly contact to the wound bed. The swelling properties (SR) of samples will be studied by immersing the samples in a solution at different periods of time at 37°C. The samples were dried until reached constant weight and cut into small piece. At predetermined time intervals, the samples were taken out from the solution and gently wiped with the paper to remove the surface solution. The samples were weighed and return to the same container until

equilibrium. The swelling ratio of these samples was calculated by following equation (2):

$$\text{Swelling ratio } (\%) = \frac{Ws - Wd}{Wd} \times 100 \qquad (2)$$

Where Ws is the weight of swollen hydrogel at different swelling time.

W_d is the weight of dry gel at different swelling time.

3.4.2.3 Equilibrium Degree of Swelling (EDS)

Samples of hydrogel dressings were weighed (w_1) and put in distilled water. After 72 h, the samples were removed from the water, surface wiped with blotting paper and weighed (w_2). The % water uptake were calculated by Eq. (3).

$$\%EDS = \frac{W2 - W1}{W1} \times 100 \qquad (3)$$

3.4.2.4 Moisture Retention Capability

A piece of hydrogel, with thickness of 4 mm,and weight about 0.5 g, was put into a plastic cup and then left in the room temperature. The sample was weighed at initiation (m_0) and different intervals (m_t). moisture retention capability was measured by the water losing rate and the ratio of water holding in hydrogel piece (R_h), which was calculated as follows (4):

$$Rh\ (\%) = \left(\frac{mt}{m0}\right) \times 100 \qquad (4)$$

3.4.2.5 Measurement of Water Vapor Transmision Rate

The moisture permeability of hydrogels was determined by measuring the water vapor transmission rate (WVTR) across the material according to monograph of the European Pharmacopeia. It consist of measuring the weight loss of a bottle which contain 10 ml of water. The hydrogels were mounted on the mouth of cylindrical glass bottle (13 mm diameter) containing 10 ml water with negligible water vapor transmittance. The material was fastened using Paraffin film across the

edges to prevent any water vapor loss through the boundary and place in an oven at 35°C for 24 h. The WVTR was calculated by using the following formula (5):

$$WVTR = \frac{(Wi-Wt)}{A\times 24} \times 10^6 \ g/m^2 \ h \qquad (5)$$

Where WVTR was expressed in g/m^2h, A is the area of a bottle mount (mm^2), *Wi* and *Wt* are the weight of bottle before and after placed in oven, respectively. Experiments were done in triplicate.

3.4.2.6 SEM Observation

Scanning Electron Microscope (SEM) observation was conducted on a JEOL JSM-5200 with an acceleration voltages of 15 kv, magnification in the range of 1000-5000x. The samples were coated with a thin layer of Au by an Ion Sputtering Device prior to SEM observation. SEM images of the samples were taken to observed any change in surface morphology.

3.4.2.7 Release of Chitin Oligomers

Release of chitin oligomers from reinforced hydrogels was evaluated using phosphate buffer saline (PBS, pH = 7.4) and the buffer solution containing 0.4 mg mL^{-1} of lysozyme (pH =7.4). First, the hydrogel samples were dried in an oven under 60 °C to gain a constant weight before determining theirs biodegradability, after that the samples were placed in 10 mL of neat and lysozyme containing buffer solutions in a shaking bath at 37 °C. After the evaluation time, samples were removed and dried in an oven at 60 °C and weighed after they were completely dried. The percentage of weight loss of the samples was calculated according to equation Eq. (6).

$$\text{weight loss } (\%) = \frac{Wo-Wt}{Wo} \times 100 \qquad (6)$$

Where Wo is the dried weight of samples in buffer solution at evaluation time and Wt is the dry weight of samples after enzymatic hydrolysis in buffer solution containing lysozyme at the same evaluation time.

3.4.2.8 Tensile Stregth and % Elongation

Tensile strength and % Elongation measurements of hydrogel samples were carried out on Lloyd Universal testing machine model LRX with a 500 N load cell at room temperature and cross-head speed of 50 mm/min were used. The Sample strips of 5 cm ×1 cm × 0.2 cm were cut from hydrogel. The data was evaluating to tensile strength (MPa) and % Elongation at break.

3.4.2.9 Indirect Cytotoxicity Test

An indirect cytotoxic test was conducted on the neat AMPS-Na^+ hydrogels and chitin whiskers-reinforced AMPS-Na^+ hydrogrls by using L929 mouse fibroblasts cells. L929 was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum (FBS; BIOCHROM AG, Germany), 1% L-glutamine (Invitrogen Corp., USA), and 1% antibiotic and antimycotic formulation (containing penicillinGsodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA)). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The MTT assay was carried out to confirm the viability of cells. First, extraction medium was prepared by immersing hydrogel in wells of a 12-well culture plate in a Serum-free media (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) at ratio of 10 mg of hydrogel : 1 ml of SFM for 7 days, then the hydrogels were removed and the extracts were stored for evaluation of cytotoxicity. L929 were separately cultured in wells of a 48-well culture plate in serum-containing DMEM for 16 h to allow cell attachment on the plate. The cells were then starved with SFM for 24 h, after which time the medium was 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 via 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 per well of MTT solution at 0.5 mg/ml for a 48-well culture plate. Secondly, the plate was incubated for 1 h at 37°C. The solution is then aspired and 900 µl per well of dimethylsulfoxide (DMSO) containing 125 µl per well of glycine buffer (pH=10.5) is added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution at 570 nm is measured using a Thermospectronic Genesis 10 UV/Visible spectrophotometer.