CHAPTER III

EXPERIMENTAL

3.1 Materials

3.1.1 Polymers

- Poly(vinyl alcohol), 99+ % hydrolyzed ; Sigma-Aldrich,USA
- Chitosan, 85 % DD , Medium molecular weight ;Sigma-Aldrich,USA

3.1.2 Chemicals

- N-(3-chloro-2-hydroxypropyl) trimethylammonium chloride solution, 60 wt % in H_2O ; Sigma-Aldrich, USA
- Sodium tripolyphosphate (TPP), technical grade 85 % ; Sigma-Aldrich, USA
- Tetracycline hydrochloride (TC) ; SRL, India
- Agar powder for bacteriology (TSA) ; SRL, India
- Tryptone Soya Broth (TSB) ; SRL, India
- Tween 80 ; RFCL Limited, India
- Iodine ; Sigma-Aldrich, USA
- Sodium hydroxide ; RCI Labscan, Thailand

3.1.3 Solvents

- Acetic Acid ; J.T. Baker, USA
- Methanol ; RCI Labscan, Thailand
- Acetone ; RCI Labscan, Thailand

3.1.4 Material Used for Bacterial Culture

3.1.4.1 Model Bacterial

Escherichia coli (E.coli) and *Staphylococcus aureus (S.aureus)*were obtained from the Department of Biology, Faculty of Science, Chulalongkorn University (Thailand)

Enterococcus faecium (Ent.Faecium) was obtained from the Nopparat Rajathanee Hospital (Thailand)

3.1.4.2 Bacterial Culture Medium

Bacterials were cultured in Tryptone Soya Broth (TSB; SRL, India), prepared by using 3% TSB in 100 mL of Distilled water and Agar plates was prepared by using Agar powder for bacteriology (TSA; SRL, India), which contained 3 % TSB and 1.5 % TSA in 100 mL of Distilled water.

3.1.5 Materials Used for Cell Culture

3.1.5.1 Model Cells

Human fibroblasts cells (FB) and *mouse fibroblasts (L929)* were obtained from the Department of Anatomy, Faculty of Density, Chulalongkorn University (Thailand)

3.1.5.2 Cells Culture Medium

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS; BIOCHROMAG), 1% L-glutamine (Invitrogen Corp.), and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)]

3.1.5.3 Material Medium

Materials were immersed in serum-free medium (SFM) which contained DMEM without phenol red, 1% L-glutamine, 1 % lactabumin, and 1% antibiotic and antimycotic [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)].

3.1.5.4 Cell Culture Study

- Ethanol : J. T. Beaker, USA
- 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) : USB Corporation, USA
- DMEM without phenol red : GibThai Co., Ltd.
- Dimethylsulfoxide (DMSO) : Sigma-Aldrich, USA
- Trypsin-EDTA solution : GibThai Co., Ltd.

- L-glutamine : GibThai Co., Ltd.
- Fetal Bovine Serum : Sorali, Brazil
- Glycine : Sigma-Aldrich, USA
- Lactabumin : GibThai Co., Ltd.

3.2 Equipment

3.2.1 Petri Dish (10 cm in diameter)

3.2.2 Fourier Transforms Infrared Spectrometer (FTIR)

Fourier transforms infrared spectrometer (FTIR; Thermo Nicolet Nexus 670) was used to determine the functional groups on quternized chitosan.

3.2.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR; Bruker AVANCE 400 MHz spectrometer) was used to confirm the synthesis of quaternary ammonium chitosan and to calculate the degree quaternization of chitosan.

3.2.4 UV-Visible Spectrophotometer (UV-vis)

UV-Vis spectrophotometer (UV-vis; Shimadzu UV-1800) was used to confirm the loading of tetracycline (TC) into quaternized chitosan nanoparticles.

3.2.5 Field Emission Scanning Electron Microscope (FESEM)

Field emission scanning electron microscope (FESEM; JSM-7001F) was used to observe the surface morphologies and shape of the QCh/TC_NPs.

3.2.6 X-ray Diffraction (XRD)

X-ray Diffraction (XRD; Rigaku/DMAX 2200HV) was used to determine the packing structures of nanoparticle and to confirm the loading of tetracycline (TC) into quaternized chitosan nanoparticles.

3.2.7 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS; Nanosizer model S4700, Malvern Instrument, UK) was used to measure particles size of quternized chitosan and quternized chitosan loaded tetracycline.

3.2.8 Zeta Potential Measurement

Zeta potential measurement (Zetasizer model S4700, Malvern Instrument, UK) was used to measure the zeta potential of quternized chitosan.

3.2.9 Micropalted Reader

Microplate reader model Infinities 200 PRO NanoQuant Multimode Microplate Reader from Tecan AG was used to determine the optical density of the samples in MTT assay.

3.3 Methodology

3.3.1 Preparation of Quaternized Chitosan (QCh)

QCh was prepared by method of (Sajomsang *et al.*, 2009).Briefly, chitosan 1 g was dissolved in 50 mL of 1 % (w/v) acetic acid and stirred at room temperature overnight. And then, chitosan solution was added into 2% (w/v) of Na_2CO_3 in H_2O/MeOH after that filtration and used to next step. The solution of N-(3-chloro-2-hydroxypropyl) trimethylammonium Chloride 40 mL (NTMC) was added to the reaction bottle, and the pH of the solution was raised to 8 by using 20% (w/v) NaOH. And then, 0.25 g of iodine was added with the regenerated chitosan into reaction bottle, stirred for 48 h at room temperature, added distilled water, and raised the temperature to 60 ^{0}C for 24 h. The solution was dialyzed with distilled water for 48 h. The dialyzed solution vacuum using a rotary evaporator, then precipitated in acetone and dried at room temperature overnight under nitrogen gas.

3.3.1.1 Characterization of Quaternized Chitosan (QCh)

- ^{1}H NMR Analysis

The ^{1}H NMR spectra were measured on a Bruker AVANCE 400 MHz spectrometer. All measurements were performed at 300K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. 1% (v/v) CD_3COOD in D_2O was used to dissolve 5 mg of chitosan and quaternized chitosan. ^{1}H NMR-spectroscopy was applied to identify the structure; the degree of quaternization (%DQ) was calculated by Eq. (1).

$$DQ\ \% = [1 - \left(\frac{\frac{NHAc}{3}}{b}\right)] \times 100 \qquad (1)$$

where NHAc is the integral area of acetyl protons of chitin and b is the integral area of methine proton of NTMC substituted derivatives.

- FTIR Analysis

The FTIR spectra were measured in transmission mode on Thermo Nicolet Nexus 670 for characterizing the occurrence of the specific chemical groups. FTIR spectra were scanned in the range of the wavenumber from 4000 to 400 cm^{-1} by using OMNIC. In all cases, the spectra were collected using 32 scans with a resolution of 4 cm^{-1}.

- Zeta potential measurement

The zeta potential was measured by using zetasizer model S4700, Malvern Instrument, UK, for determination of electrostatic potential on samples surface in distilled water at 25 ^{0}C.

- Estimation of Water Solubility

The pH dependence of sample water solubility was evaluated using turbidity measurements. The QCh (0.2 g) were dissolved in 1% acetic acid (100 ml) and NaOH (2 M) was added stepwise into derivative solution. The transmittance of the solution was recorded by using UV-vis spectrophotometer at 608 nm.

3.3.2 Preparation of Quaternized Chitosan Loaded Tetracycline Nanoparticles (QCh/TC_NPs)

The QCh nanoparticles were prepared based on the ionic cross-linking of QCh with TPP. The QCh solution was prepared by dissolving QCh 25 mg in 10 ml of distill water at room temperature until clear solution. Tween 80 (0.25 g) was added to the solution and stirred at 60 ^{0}C for 1 h and then cooled to room temperature. Tetracycline was added to the solution and stirred for 20 min to get homogeneous solution (QCh to drug ratios of 1:0, 1:1, 1:2, 1:3, 1:4 and 1:5. The TPP solution (40 ml, 0.5 % w/v) was gradually dropped (2 mL/min) into the QCh/TC after that keep stirred 20 min for completely formation. The nanoparticles were collected by centrifugation at 10,000 rpm for 30 min at 20 ^{0}C and washed the nanoparticles by water several times for removing free tetracycline. The nanoparticles redispersed in 10 mL of distill water and stored at -20 ^{0}C.

3.3.2.1 Determination of Tetracycline Encapsulation Efficiency

TC was standardized in water at 270 nm using UV–vis spectrophotometer (UV-1800) and standard graph with standard equation was

obtained. The encapsulation efficiency of TC in QCh nanoparticles was determined by separating free TC molecules which untapped in QCh_NPs in the supernatant and then measured absorbance of untapped TC in water. Encapsulation efficiency was calculated as **Eq. (2)**

Encapsulation efficiency (%)

$$= \frac{\text{Total amount of TC added} - \text{Free TC in supernatant}}{\text{Total amount of Tet added}} \times 100 \qquad (2)$$

3.3.2.2 Characterizations of QCh/TC_NPs

The particles size of QCh and QCh/TC_Nps were determined at 25^{0}C by using dynamic light scattering (model S4700; Malvern Instrument, UK. The surface morphology of nanoparticles was carried by using field emission scanning electron microscopy (FESEM; JSM-7001F) and the nanoparticles suspensions were spread on a glass plate and dried at room temperature. The dried nanoparticles were coated with gold metal under vacuum and then examined (Ji *et al.*, 2011). The chemical interactions of TC with QCh_Nps were studied by using Fourier transform infrared spectrophotometer (Thermo Nicolet Nexus 670). The success of TC encapsulation was confirmed by using UV-Vis spectrophotometry (UV-1800) and X-ray diffractometer (Rigaku/DMAX 2200HV.) were recorded over a 2θ range of 10-50^0 with step angle of 10 ^{0}C/min.

3.3.3 Preparation of Wound Dressing

Poly (vinyl alcohol), 5 % w/v was dissolved in hot water at 90 ^{0}C for 4 h and then cooled down. After that, the QCh/TC_NPs suspensions were added in PVA solution for casting on the petri dish and were dried under atmosphere for 2 d.

3.3.3.1 In vitro Release of Wound Dressing

The PVA films were immersed at desired time of 1, 2, 3, 4, 24, 48, 72, 96, 120, 144 h in 25 mL PBS buffer (pH 7.4) and acetate Buffer (pH 5.5). Tetracycline released in supernatant was measured by UV-vis spectrophotometry in PBS buffer and acetate buffer as baseline at 270 nm and 276 nm, respectively. Based on the standard equation obtained for TC from the standard graph and concentrations

of TC released at different time were calculated. All the experiments were done in triplicate. The percentage of TC released in the supernatant was calculated based on the **Eq. (3)**

$$\text{Drug release (\%)} = \frac{\text{Released TC at a desired time}}{\text{Total amount of Tet entrapped within nanoparticles}} \times 100 \quad (3)$$

3.3.3.2 Antibacterial Studies

- MIC and MBC Evaluations

The minimum inhibitory concentration (MIC) of tetracycline was determined by a broth dilution method (Keawchaoon *et al.*, 2011). Three of bacteria, *E.coil*(-), *S.sureus* (+), *Ent.faecium* (+) , were used testing. All bacterial were inoculated in TSB medium at 37 ^{0}C for 24 h, after that the concentration of culture suspensions were measured to 10^8 CFU/mL in 0.85 % NaCl by comparison with McFarland turbidity standard 0.5.Tetracycline 10.0 mg/mL was prepared in microtube in 1 mL of TSB medium and then diluted concentration to next microtube that has 0.5 mL TSB for getting haft concentration of tetracycline. Bacterial suspensions (0.5 mL) were add in the tetracycline solution of each microtube and then inoculated at 37 ^{0}C for 24 h. After that, MIC was determined as the concentration of tetracycline in the tube without turbidity and containing the lowest concentration. The minimum bactericidal concentration (MBC) was evaluated by transferring 100 µL of all tubes without turbidity to agar plates and incubated at 37 ^{0}C for 24 h. Moreover, the MBC was determined as the concentration of tetracycline in the tube related to the agar plate without bacterial growth and containing the lowest concentration.

- Agar Diffusion Tests

The agar diffusion tests (AATCC 147) was method used to determine the ability of diffusible antimicrobial of wound dressing. In this method, the PVA films were cut into circular discs (1.2 cm in diameter) and then the agar

plates were prepared by using agar power for bacterial. The specimens of PVA films were sterilized by exposing to UV radiation for 30 min before they were placed in intimate contact with agar plate and incubated at 37 ^{0}C for 24 hours. The clear zone of interrupted growth underneath and the size of clear zone indicated antibacterial activity of the PVA film. The average zone of inhibition zone was calculated by the following Eq.

$$W = \frac{T-D}{2} \tag{4}$$

Where T is total diameter of test material and clear zone (in mm) and D is diameter of the test material (in mm).

- Dynamic Shake Tests

The dynamic shake tests (AATCC 100) was method used to evaluate quantitative values on the antimicrobial material. The concentrate of bacterial culture was diluted to 10^6 CFU/mL in 0.85 % NaCl or Peptone medium. Each PVA film (about 0.02 mg) was transferred to flask containing 100 mL of 10^6 CFU/mL. All flasks were shaken for 1,3 and 24 hours at 110 rpm and at 37^{0}C.At desired time,100 µL of solution was diluted into 10^5, 10^4, 10^3, 10^2, 10^1 CFU/mL in microtubes and 100 µL of each dilution was plated in agar plates. The agar plates were incubated at 37^{0}C for 24 h and surviving bacterial were counted. The antimicrobial activity was expressed in percentage reduction of the bacterial after contact with the PVA films compared to the number of bacterial surviving after contact with the control. The percentage reduction was calculated by using the following Eq.

$$\text{Reduction } \% = \frac{B\text{-}A}{B} \times 100 \tag{5}$$

Where "A" were the surviving bacterial (CFU/ml) after contact with PVA films and "B" were the surviving bacterial (CFU/ml) after contact with control on specific time.

3.3.3.3 Cytotoxicity Studies

The human fibroblasts cells (FB) and *mouse fibroblasts (L929)* were used to determine the biological response of PVA film loaded QCh/TC_NPs. Cells were cultured in α-MEM medium supplement with 10% FBS, 1

% L-glutamine and 1 % antibiotic and antimycotic. The medium was changed every 2 days and the cultured cells were maintained at 37 ^{0}C in a humidified atmosphere containing 5% CO_2

- Material Preparation for Cells Studies

The 12 mm-diameter circular of PVA films were sterilized by UV radiation for 30 min before were put into 24-well tissue-culture polystyrene plates (TCPS; Biokorn Systems, Ppland), which were sterilized with 70% ethanol. The specimens were immersed in SFM medium for 1 day and 3 day. *L929 cells* and *FB cells* from the culture were trypsinized [0.25 % trypsin containing 1 m MEDTA (Invitrogen Crop., USA)] and were counted by a hemacytometer (Hausser Scientific, USA).Cells were added at a density about 40,000 cells/cm^2 on the specimens and empty wells of TCPS that act as control. The culture cells were maintained in an incubator at 37 ^{0}C in a humidified atmosphere containing 5% CO_2.

- Indirect Cytotoxicity Evaluation

Indirect cytotoxicity test was conducted on TCPS, the PVA films in ratio of QCh: TC of 1:0, 1:3, 1:4, 1:5 and pure PVA. Firstly, the extraction medium was prepare by immersing PVA films in a serum-free medium (SFM; containing DMEM, 1 % L-glutamine, 1 % lactabumin, and 1 % antibiotic and antimycotic). Then placed under 5 % CO_2 at 37 ^{0}C in 24-well plate for 1 and 3 days. 40,000 cells of *L929 cells* and *FB cells* were separately cultured in other 24-well plate in 500 µL DEME to allow cell attach on the plate for 1 day. The cells were starved with 300 µL SFM for 1 day and then the culture medium was replaced with the 300 µL of extraction medium. After 24 hour, cells cultured in extraction medium were carried by MTT assay for quantify the amount of the viable cells.

- MTT Assay

MTT assay was used to determine the number of viable cells, 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 crystal formed was relation to the quantity of viable. Culture medium was remove out in each well and incubated in 400 µL MTT-solution at 37 ^{0}C for 30 minutes. After that, MTT solution was removed and buffer

solution was replaced which containing 900 μL/well dimethysulfoxide (DMSO) and 125 μL/well glycine buffer to dissolve the formazan crystal then the solution was shaken for 10 min and was transferred to measure the number of viable cells by spectrophotometer (Thermospectromic Genesis10 UV-visible spectrophotometer) at absorbance 540 nm. The cell viability (%) was calculated according to the following Eq.

$$\text{Cell viability (\%)} = \frac{\text{OD 540 sample}}{\text{OD 540 control}} \times 100 \quad (6)$$

Where OD540 (sample) was the optic density obtained from the wells treated with the material, and OD540 (control), from the wells treated with DMEM.

3.4 Statistical Analysis

The experiments are repeated 3 times and the results are expressed as a mean ± standard deviation. The values of $p < 0.05$ were considered significant.