CHAPTER III

EXPERIMENTAL

3.1 Materials

Polymers

- Chitin

  Squid pens, *Loligo pealei*

Chemicals

- Curcumin , 95.0% purity (Fluka)
- Tween 20 (Labchem)
- Ethanol (J.T.Baker)
- Sodium acetate (Ajax Chemicals)
- Sodium chloride (Lab-Scan)
- Di-sodium hydrogen orthophosphate (Ajax Finechem)
- Sodium dihydrogen orthophosphate (Ajax Finechem)
- HCl (Lab-Scan)
- NaOH(Lab-Scan)

3.2 Equipments

1. Fourier transform infrared spectrometer (FTIR)
2. Scanning electron microscope (SEM)
3. UV-vis spectrophotometer
4. Optical microscope
5. Vacuum oven
6. High speed blender (Phoenix)
7. Shaking incubator
8. Micropipette
9. Glass mold
10. Hot plate

11. Petri dish
12. Volumetric flasks
13. Vials

3.3 Methodology

3.3.1 Chitin Preparation

a. Chitin was prepared by immersing grinded squid pen in 1 M hydrochloric acid (HCl) for 2 days. The ratio of grinded squid pen to HCl solution was 1 g to 10 ml of the HCl solution and a fresh solution should be made everyday.

b. After decalcification, the deproteinization process was done by using a 4% sodium hydroxide (NaOH) solution. The obtained flakes were immersed in a NaOH solution in the same ratio that was used in decalcification, for 1 day. The mixture was finally produced by heating the mixture on a hot plate at 80°C for 4 h.

c. The obtained β-chitin flake was washed and dried in an oven at 60°C for 24 h.

3.3.2 Fabrication of Chitin Sheets

a. One gram of chitin flake, 400-500 μm, was suspended in 50 ml of distilled water and agitated by a high speed blender for 60 sec.

b. The suspension was then heated on a hot plate at 50°C for 20 min followed by repeated agitation in the high speed blender.

c. The processes of agitation and heating of chitin suspension were repeated for 7 times in order to obtain a fibrous form of chitin in the suspension.

3.3.3 Incorporation of Curcumin into the Chitin Sheets

3.3.3.1 Preparation of High-dose Curcumin-loaded Chitin Sheets

a. Before the first agitation of chitin suspension in the high speed blender, 0.5% v/v of Tween®20 based on the total amount of the chitin suspension, was added into the chitin suspension.

b. At the same time, curcumin powder in various amounts was also added into the suspension to achieve curcumin content in the amounts of 1, 5, 10, and 20 wt% based on the weight of chitin.

c. The obtained suspension was agitated for 60 sec repeated for 7 times before degassing under a vacuum.

d. Finally, the suspension was poured onto a glass mold, and air dried under a dark laminar flow hood.

3.3.3.2 Preparation of Low-dose (fully dissolved) Curcumin-loaded Chitin Sheets

a. Dissolve 0.04 g of curcumin powder in 20 ml of distilled water in the presence of Tween 20, 0.5 ml.

b. Pour curcumin solution into the chitin suspension followed by agitation in a high speed blender for 60 sec.

c. The obtained mixture was agitated for 60 sec repeated for 5 times before degassing under a vacuum.

d. Finally, the suspension was poured onto a glass mold, and air dried under a dark laminar flow hood.

3.3.4 Characterizations

3.3.4.1 Degree of Deacetylation

a. Fourier transform infrared spectra of films were provided from a Thermo Nicolet 670 in the range of 400-4000 cm^{-1} with scans at a resolution of 2 cm^{-1}. The degree of deacetylation was calculated based on the

FTIR spectra using the following Eq. 1(Sannan *et al.*, 1978; Baxter *et al.*, 1992):

$$\% \text{ DD} = 100 - [(A_{1655} / A_{3450}) \times 155] \quad (1)$$

where %DD is the degree of deacetylation, A_{1655} is the absorbance at 1655 cm^{-1}, and A_{3450} is the absorbance at 3450 cm^{-1}.

3.3.4.2 Morphology Observation

a. The surface morphology of the curcumin-loaded chitin sheets was examined by scanning electron microscope (SEM) conducted on a JEOL JSM- 5200 with an acceleration voltage of 10 kV, magnification in the range of 75-3500x.

b. The samples were coated with a thin layer of Au by an ion sputtering device prior to the SEM observation.

3.3.4.3 Interaction between Curcumin and Chitin Sheets

a. The interaction between chitin and curcumin were determined by Fourier transform infrared (FTIR) spectra, Thermo Nicolet 670, in the range of 400-4000 cm^{-1} with scans at a resolution of 2 cm^{-1}.

3.3.5 Releasing Behaviour of Curcumin from Curcumin-loaded Chitin Sheets

3.3.5.1 Preparation of the B/E/T Calibration Curve

a. Due to the solubility limitation of curcumin in the acetate buffer solution, the B/E/T medium (94% v/v acetate buffer with 2% v/v Tween 20 and 4% v/v ethanol) was used to determine the calibration curve.

3.3.5.2 Determination of Actual Curcumin Content

a. The actual amount of curcumin in the curcumin-loaded chitin sheets was determined by immersing each specimen (circular disc; ~1.5 cm in diameter) in 2 ml of 2:1 (v/v) of Tween 20/ethanol (T/E solution).

b. After 24 hours, the actual amount of curcumin was measured by a Shimadzu UV-2550 UV-vis spectrophotometer at the wavelength of 424 nm. The actual amount of curcumin in the curcumin-loaded chitin sheets was back-calculated from the obtained data against a new predetermined calibration curve of curcumin in the T/E solution.

3.3.5.3 Curcumin-Release Assay

a. By the total immersion assay, acetate buffer was chosen as a releasing medium stimulating the human skin, pH 5.5. Each specimen (circular disc; ~1.5 cm in diameter) was immersed in 5 ml of the medium at the physiological temperature of 37 °C.

b. At a specified immersion period ranging between 0 to 72 h (4320 min), one ml of a sample solution was dipped out and an equal amount of the fresh medium was then added back to the vial.

c. The amount of curcumin in the sample solutions was determined using the UV-vis spectrophotometer at the specified wavelength of 424 nm. The obtained absorbance values were calculated by using the following Eq. 2:

$$\% \text{ Release of curcumin} = \frac{[\text{released curcumin}]_{t=t}}{[\text{actual amounts of curcumin}]_{t=0}} \times 100 \quad (2)$$

where t is the specific immersion times. The experiments were carried out in triplicate and the results were reported as average values.

3.3.6 Stability of Curcumin at the Neutral Buffer Solution

3.3.6.1 Stability of Ethanol-soluble Curcumin in Phosphate Buffer, pH 7.2

a. Phosphate buffer (pH 7.4) was chosen as a neutral medium stimulating the physiological condition at 37°C. Curcumin was weighed accurately to give a final concentration of 0.02 mg/ml with absolute ethanol.
b. Drop 1 ml of ethanol-soluble curcumin into the vial containing 9 ml of phosphate buffer. Then place the vial in a shaking incubator at 37°C.
c. At specific incubation times, the solution was removed and suddenly determined the absorption spectra conducted by the UV-vis spectrophotometer, spectrum scanning modes in a range of 200-700 nm.

3.3.6.2 Stability of Curcumin Released from Chitin Sheets in Phosphate Buffer, pH 7.2

a. Immerse a specimen (circular disc; ~1.5 cm in diameter) in the vial containing 5 ml of phosphate buffer solution, pH 7.4. Then place the vial in a shaking incubator at 37°C.
b. After 3 days of incubation time, the sample was removed out. The remained buffer solution was determined using the UV-vis spectrophotometer, spectrum scanning modes in a range of 200-700 nm.
c. The remained buffer solution was put back in the shaking incubator for 7 days before determining the absorption spectra using the spectrum scanning modes in a range of 200-700 nm.

3.3.6.3 Stability of Curcumin Incorporated with Chitin Sheets in Phosphate Buffer, pH 7.2

a. After incubating in phosphate buffer for 7 days, curcumin-loaded chitin sheets was removed out and immersed in the T/E solution for 24 hours.
b. Then the extracted solution was determined using scanning modes of the UV-vis spectrophotometer

observing the absorption spectra in a range of 200-700 nm.

c. Prolong the immersion time in the T/E solution for 7 days, before repeating the scanning modes in a range of 200-700 nm.

3.3.7 Direct Cytotoxicity Test

3.3.7.1 Cell Culture

a. The target cells, L929 (mouse connective tissue ECACC Cat. No. 85011425) and human dermal fibroblasts at passage 6, were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100 unit/ml penicillin and streptomycin. The cells were incubated at 37 °C in a fully humidified, 5% CO_2: air atmosphere.

3.3.7.2 Neutral Red Assay

a. The neutral red assay, which was qualitative useful for differentiating viable and non-viable cells, conformed to ISO10993-5 and USP24 with slight modifications. One piece of 1×1 cm of each sample film was put onto a 35-mm tissue culture dish prior the seeding of the cells at a density of 3×10^5 cells/dish and then incubated for 24 hours.

b. Cell morphology and the toxicity were evaluated after a 24-hours exposure to the cells. The cells were stained with 0.01% neutral red in PBS for membrane integrity.

c. The toxicity was assessed by microscopic examination compared to control cells without sample.