CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemicals

In the current work, chitin was prepared from the shells of *Metapenaeus dobsoni* shrimp, which were supported from Surapon Foods Public Co., Ltd. (Thailand).Natural rubber latex from Hevea brasiliensis trees (Commercial grade., Chantaburi, Thailand). Analytical grade anhydrous sodium hydroxide pellets and analytical grade hydrochloric acid were purchased from Labscan. Pluronic F-68 was purchased from Sigma-Aldrich. Glacial acetic acid (J.T.Baker, Analytical grade), Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), Modified Lowry Protein Assay Kit (Thermo Scientific), Ammonium hydroxide having 28.0-30.0 wt% NH_3 in water (J.T. Baker, Analytical grade) were used.

3.2 Methodology

3.2.1 Preparation of Deproteinized Natural Rubber Latex

The natural rubber latex was centrifuged at 12000 rpm for 5 min at 25°C to remove the serum. The natural rubber was then suspended in 4 % (v/v) NH_4OH solution. The protein residuesin the natural rubber latex was removed by the addition of 0.04 % (w/v) protease. The suspension was shaken in an incubator at 100 rpm for 24 hr at 37 °C. After that, the suspension was centrifuged at 12000 rpm for 5 min and the supernatant was discharged. The deproteinized natural rubber latex was preserved in a 4 % (v/v) NH_4OH solution.

3.2.2 Preparation of chitin

The preparation of chitin consists of two steps that are the demineralization and the deproteinization. The demineralization was performed by adding 1N HCl (10 L) to dried shrimp shells (1000 g) and stirred at 25°C for 2 days with the daily change of the exhausted 1N HCl. The demineralized shrimp shells

were neutralized by deionized water and dried at 60°C for 48 hr. Then, the demineralized shrimp shells were further deproteinized by adding 4% w/v NaOH (the ratio of NaOH to shrimp shell was 10:1) and stirred at 80°C for 4 hr. Finally, the chitin was filtrated, neutralized by distilled water, and dried at 60°C for 24 hr.

3.2.3 Preparation of chitin whisker suspension

Chitin whisker suspension was prepared by acid hydrolysis of chitin. Chitin flakes were hydrolyzed with 3N HCl at which the ratio of chitin to HCl was 1g of chitin to 30mL of HCl, and the suspension was stirred at 104°C for 6 hr. After acid hydrolysis, the suspension was diluted with distilled water, followed by centrifugation at 12,000 rpm for 10 min. Then, the suspension was dialyzed against distilled water until neutral. The suspension was kept in a refrigerator before use.

3.2.4 Preparation of coconut oil/pluronic emulsion

Pluronic F-68 was dissolved in distilled water. Then, pluronic F-68 solution and coconut oil were mixed together by varying the ratio of pluronic to coconut oil to be 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, and 80/20 by using the high speed mixer for 3 min at 11200 rpm.

3.2.5 Preparation of natural rubber/chitin whisker film containing coconut oil in pluronic micelles

The preparation of the natural rubber/chitin whisker nanocomposite films containing coconut oil in pluronic micelles consist of two steps. First, the coconut oil/pluronic emulsion was stirred with deproteinized natural rubber latex at the ratio of emulsion to latex to be 30/70 at 1000 rpm for 3 min at 25°C. Then, the chitin whisker suspension was poured and shaken in an incubating shaker at 1000 rpm for 3 min at 25°C. The chitin whisker content in the nanocomposite films were varied to be 2.5%wt, 5%wt and 10%wt. The mixtures were casted on the petri dish, kept at 25°C for 2 days and then dried at 45 °C for 2 days. The composition of materials was shown in table 3.1.

Table 3.1 The composition of materials

Sample		Pluronic (g)	Oil (g)	NR* (g)	Chitin whisker (g)
A Pluronic:Oil (weight:weight) 1:27	A1	0.50	13.59	21	2.5
	A2	0.50	13.59	21	5
	A3	0.50	13.59	21	10
B Pluronic:Oil (weight:weight) 1:18	B1	0.60	10.87	21	2.5
	B2	0.60	10.87	21	5
	B3	0.60	10.87	21	10
C Pluronic:Oil (weight:weight) 1:11	C1	0.70	8.15	21	2.5
	C2	0.70	8.15	21	5
	C3	0.70	8.15	21	10

* If the content of natural rubber is more than 21 grams, it will occur phase separation before the film is dried.

3.2.6 Preparation of acetate buffer

Acetate buffer was chosen to simulate human skin pH condition of 5.5. To prepare 1000 ml of the acetate buffer solution, 150 g of sodium acetate was dissolved in distilled water. 15 ml of glacial acetic acid was then added very slowly into the aqueous sodium acetate solution.

3.3 Characterization

3.3.1 Fourier Transform Infrared Spectrometer (FT-IR)

The natural rubber, deproteinized natural rubber, chitin and chitin whisker films were characterized for the functional groups by a FT-IR spectrometer

(Thermo Nicolet, Nexus 670) in transmittance mode with 64 scans and a resolution of ± 4 cm^{-1} over the wavenumbers range of 4000-700 cm^{-1}.

3.3.2 Transmission Electron Microscopy (TEM)

The transmission electron microscope observations were observed by JEOL model JEM 2100 using an operating voltage of 200 kV. The samples were prepared by air-drying the particles onto a carbon-coated copper grid and air-dried.

3.3.3 Scanning Electron Microscopy (SEM)

The nanocomposite films were examined for the morphological structure by the scanning electron microscope (Hitachi, S-4800). The samples were put on the holder with an adhesive tape and coated with a thin layer of platinum. The scanning electron images were investigated by using an acceleration voltage of 2 kV with a magnification in 3 kX.

3.3.4 UV-VIS Spectrophotometer

The Ultraviolet-Visible (UV-VIS) Spectrophotometer (Tecan, Infinite M200) was used for colorimetric determination of the protein content of raw natural rubber and deproteinized natural rubber based on the modified Lowry method. Both raw natural rubber and deproteinized natural rubber films were soaked in tris-HCl buffer pH 7.4 for 2 hr. Each sample was added into the modified Lowry reagent and Folin-Phenol reagent. The color change of samples was analyzed at the wavelength of 750 nm.

3.3.5 Mechanical Testing Machine

The mechanical properties of the nanocomposite films were investigated by Mechanical Testing Machine (Lloyd) in the pull with yield setup mode with preload of 0.1 N, speed of 50 mm/min and gage length of 4 cm. The values of stress at maximum load and elongation at break were reported.

3.3.6 Water Absorption Properties

The dimension stability of samples in wet state was also evaluated in term of degree of swelling and weight loss (Wongpanit et al., 2005). The degree of swelling was evaluated according to the following equation:

$$\text{Degree of swelling } (\%) = \frac{W_s - W_d}{W_d} \times 100$$

where W_d is the weight of the dried film prior to submersion in buffer pH 5.5 and W_s is the weight of the sample in the swollen state.

The samples were then dried in convection oven and measured the weight loss after immersion in buffer pH 5.5. The weight loss was estimated according to the following equation:

$$\text{Weight loss } (\%) = \frac{W_b - W_d}{W_b} \times 100$$

where W_b is the weight of dried film prior to submersion in buffer pH 5.5 and W_d is the weight of the dried film after submersion for a required time.

3.3.7 The micelle stability

The micelle stability of coconut oil/pluronic emulsion was measured by time that the emulsion was separation. (Pluronic:Coconut oil 20:80 30:70 40:60 50:50 60:40 70:30 80:20)

3.3.8 Coconut oil releasing test

The modified Franz-diffusion cell was used for determining the releasing characteristic of coconut oil from the nanocomposite films. The diffusion cell was filled with the acetate buffer solution pH 5.5 and the temperature of the buffer was maintained at 37°C. A piece of pig skin was mounted on the reservoir of the Franz cell with the contact to the surface of the buffer solution. Then, the nanocomposite film was placed above the pig skin. The coconut oil diffused through the pig skin into the buffer solution. A sample of 1 ml was withdrawn at various time intervals and simultaneously replaced with equal volume of fresh buffer solution. The releasing characteristic of coconut oil was determined by the UV-VIS spectrophotometer at the wavelength of 287 nm.