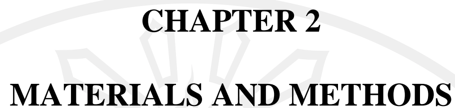

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Chemicals

- 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemical Co., St. Louis, MO, USA)
- Acrylamide (Sigma Chemical Co., St. Louis, MO, USA)
- Alpha-tocopherol (Sigma Chemical Co., St. Louis, MO, USA)
- Ammonium molybdate (Fisher Scientific UK Limited, Loughborough, UK)
- Ammonium persulfate (Amresco Inc., OH, USA)
- Ammonium thiocyanate (NH_4SCN) (Sigma Chemical Co., St. Louis, MO, USA)
- Ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA)
- Bromophenol blue dye (Amresco Inc., OH, USA)
- Calcium chloride ($CaCl_2$) (Sigma Chemical Co., St. Louis, MO, USA)
- Carbopol® 980 (Fluka, Buchs, Switzerland)
- Cholesterol (Serva Co., New York, USA)
- Concanavalin A (Sigma Chemical Co., St. Louis, MO, USA)
- Coomassie® brilliant blue G-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA)
- Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA)

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- Disodium hydrogen phosphate (Merck KGaA, Darmstadt, Germany)
- Dulbecco’s modified eagle’s medium (DMEM) (GIBCO, Grand Island, NY, USA)
- Ferric chloride ($FeCl_2$) (Sigma Chemical Co., St. Louis, MO, USA)
- Fetal bovine serum (FBS) (PAA Laboratories, GmbH, Austria)
- Glycine (BDH Chemicals Ltd., Poole, England)
- Linoleic acid (Fluka Chemicals, Gillingham, Dorset, UK)
- Mederma® (Merz Pharmaceuticals, Germany)
- Methylene blue (Fisher Scientific UK Limited, Loughborough, UK)
- Methylparaben (Gujarat Organics Limited, Mumbai, India)
- N,N’-methylenebisacrylamide (Fluka Chemicals, Gillingham, Dorset, UK)
- Penicillin-streptomycin for cell culture (PAA Laboratories, GmbH, Austria)
- Pentobarbitone sodium (Nembutal®) (Sanofi, Santé Animale Benelux, Brussels, Belgium)
- Poly (lactide-co-glycolide) (PLGA) (Wako Pure Chemical Industries Ltd., Osaka, Japan)
- Polyvinyl alcohol (PVA-403) (Kuraray Chemical Co., Ltd., Osaka, Japan)
- Propylene glycol (Jindecheng Trading Co., Ltd., China)
- Propylparaben (Gujarat Organics Limited, Mumbai, India)
- Salmon calcitonin (Kunming Jida Pharmaceutical Co., China)
- Sephadex G50 (Fluka Chemicals, Gillingham, Dorset, UK)
- Sodium azide (NaN_3) (Sigma Chemical Co., St. Louis, MO, USA)

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- Sodium cholate (NaC) (Himedia, Mumbai, India)
- Sodium deoxycholate (NaDC) (Sigma Chemical Co., St. Louis, MO, USA)
- Sodium dihydrogen phosphate (BDH Chemicals Ltd., Poole, England)
- Sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Inc., Hercules, CA, USA)
- Sodium luaryl sulfate (SLS) (Sigma Chemical Co., St. Louis, MO, USA)
- Standard bromelain (Sigma Chemicals Co., St. Louis, MO, USA)
- Standard purified papain (95%purity) (Sigma Chemicals Co., St. Louis, MO, USA)
- Sulforhodamine B (SRB) monosodium salt (Fluka Chemicals, Gillingham, Dorset, UK)
- TEMED (N,N,N',N'-tetramethyl ethylenediamine) (Fluka Chemicals, Gillingham, Dorset, UK)
- Trichloroacetic acid (Merck KGaA, Darmstadt, Germany)
- Triethanolamine (Sigma Chemical Co., St. Louis, MO, USA)
- Trifluoroacetic acid (TFA) (Merck KGaA, Darmstadt, Germany)
- Tris (hydroxymethyl)-methlamine (Sigma Chemical Co., St. Louis, MO, USA)
- Triton X-100 (BDH Chemicals Ltd., Poole, England)
- Trypsin (Gibco Invitrogen Corp., Carlsbad, CA, USA)
- Tween 61 (polyoxyethylene sorbitan monostearate) (Sigma Chemical Co., St. Louis, MO, USA)
- Other solvents (analytical grade)

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2.1.2 Cell lines

- Human skin fibroblasts (Department of Tropical Hygine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand and Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand)

2.1.3 Animals

- New Zealand white rabbits (Faculty of Agriculture, Chiang Mai University, Thailand)
- Sprague-Dawley rats (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand)

2.1.4 Equipments

- 6-Well plates (Nalge Nunc International, NY, USA)
- 96-Well plates (Nalge Nunc International, NY, USA)
- Centrifuge (Universal 32R, Hettich Zentrifugen, Germany)
- CO_2 incubator (Shel Lab, model 2123TC, SHEL LAB, VWR, USA)
- Dermal biopsy punch (Sontec Instrument, CO, USA)
- Dynamic light scattering (DLS), Zetasizer 300HSA (Nano-S, Malvern Instruments Ltd., Malvern, UK)
- Fractional collector (Foxy JR, Isco, Inc., Lincoln, USA)
- Gel documentation system and Quantity 1-D analysis software (Bio-Rad Laboratories, Inc., UK)
- High Perfomance Liquid Chromatography (HPLC, AS 1000, Thermo Finigan, USA)

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- Larminar air flow cabinet (Cytair 125, Equipments Scientifiques & Industries S.A., France)
- Lyophilizer (Christ FOC-1 Model K-40 equipment, Balzers-Pfeiffer GmbH, Asslar, Germany)
- Mexameter® (Courage & Khazaka, Cologne, Germany)
- Microplate Reader (Model 680, BIORAD, USA)
- Modified vertical Franz diffusion apparatus (Crown Bio Scientific, Inc., Sommerville, NJ, USA)
- Optical microscope (Olympus CHS, Olympus optical Co., Ltd., Japan)
- pH meter (Laboratory Benchtop 86502, AZ Instrument Corp., Taiwan)
- Poly acrylamide gel electrophoresis (BioRad Mini-PROTEIN III, Bio-Rad Laboratories, Inc., UK)
- Polycarbonate membrane filter with a pore size of 50 nm (Millipore, Billerica, MA, USA)
- Probe sonicator (Vibra Cell™, Sonics & Materials Inc., Newtown, CT, USA)
- Propeller type agitator with three blades (Heidon 600G, Shinto Scientific Co., Ltd., Japan)
- Reversed-phase column (Gemini-NX, 5μ C18 110A, 4.6×250 mm, Phenomenax, USA)
- Rotary evaporator (R-124 Büchi, Switzerland)
- Rotary Viscometer (VR 3000 MYR Viscometers, VISCOTECH, Spain)
- Scanning electron microscope (JSM-5900LV, JEOL, Tokyo, Japan)

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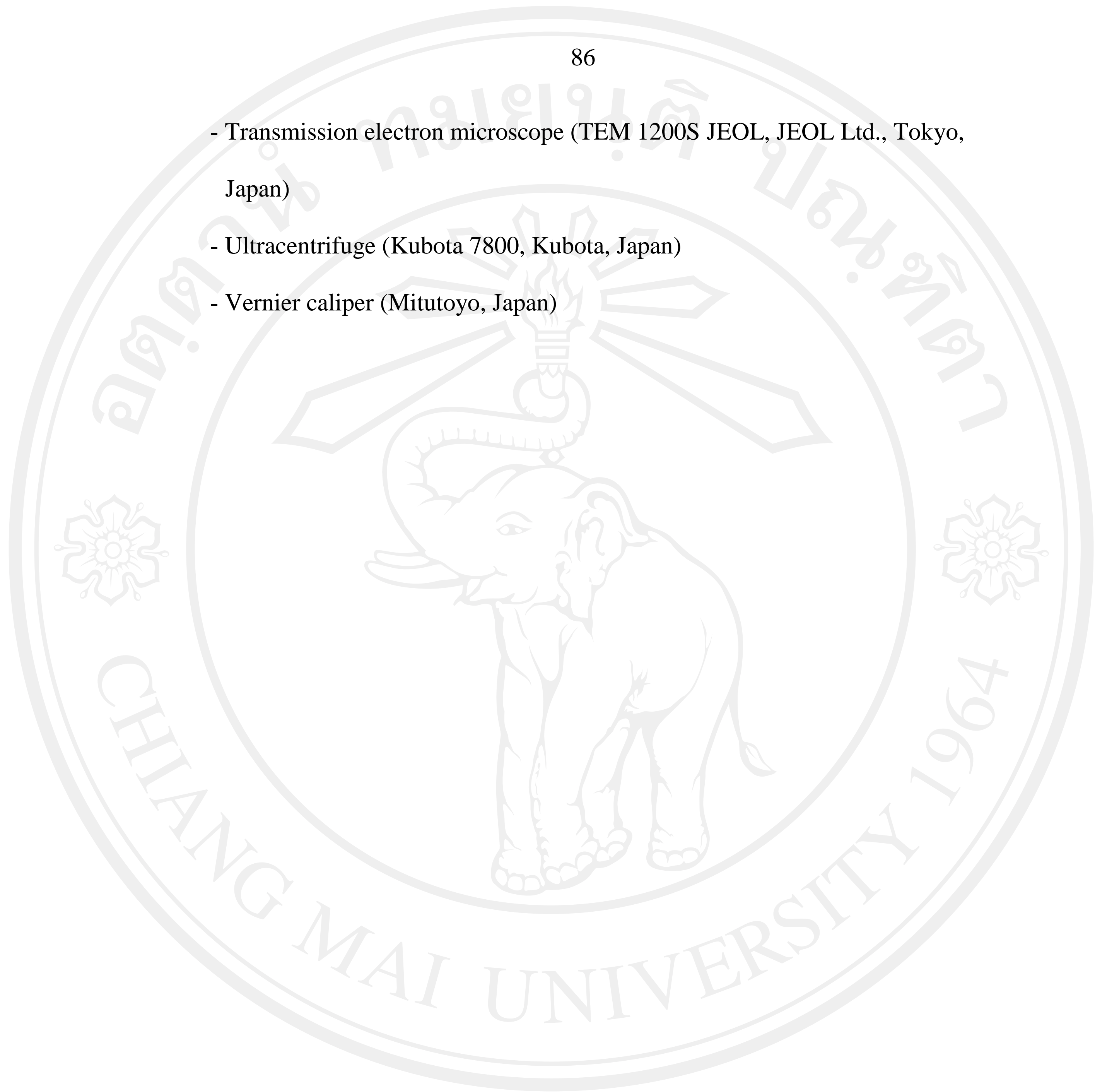
- Transmission electron microscope (TEM 1200S JEOL, JEOL Ltd., Tokyo, Japan)
- Ultracentrifuge (Kubota 7800, Kubota, Japan)
- Vernier caliper (Mitutoyo, Japan)



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2.2 Methods

The scope of the study was divided into 6 parts as the scheme in **Figure 30**

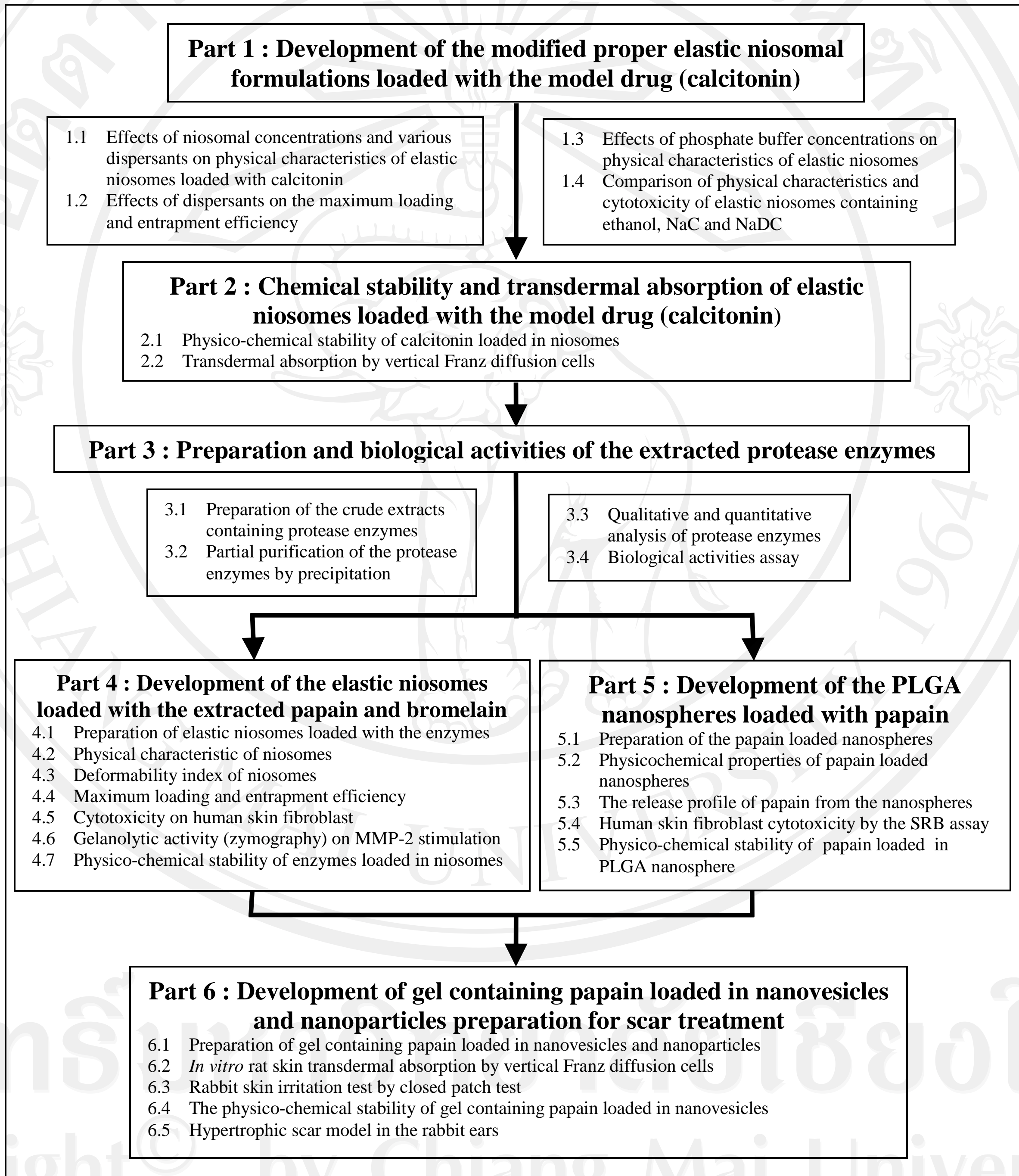

Figure 30 Scheme of the scope of the study

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Part 1 : Development of the modified proper elastic niosomal formulations loaded with the model drug (calcitonin)

1.1 Effects of niosomal concentrations and various dispersants on physical characteristics of elastic niosomes loaded with calcitonin

1.1.1 Preparation of blank niosomes and niosomes loaded with calcitonin

The non-elastic and elastic niosomes **(Table 13)** were prepared by the thin film hydration method with sonication (Manosroi et al., 2008). Briefly, cholesterol was first mixed with Tween 61 at 1:1 molar ratio of various total mixture concentrations (5, 10 and 20 mM). Then, the mixture was dissolved in chloroform/methanol (1:1) and evaporated under vacuum to get a thin film. For non-elastic niosomes, the film was hydrated with various dispersants (distilled water or 20 mM phosphate buffer, pH 7.0). For ethanol elastic niosomes, the dispersants containing 20%v/v ethanol was used. The resulting dispersion was sonicated by a microtip probe sonicator for 10 min at 4±2 °C by placing the dispersion in an ice bath.

Niosomes loaded with calcitonin were prepared with the same above method, except calcitonin at 0.22 mg/ml (equivalent to the recommended dose of calcitonin nasal spray in postmenopausal osteoporosis treatment) in distilled water or 20 mM phosphate buffer (pH 7.0) was used to hydrate the film instead of distilled water or buffer only.

1.1.2 Physical characteristics of niosomes

The appearance, color and sedimentation of the niosomal dispersion were optically observed, while the size and zeta potential values of the niosomal dispersions were determined by the dynamic light scattering with an angle detection at 173°.

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Table 13 Descriptions of various niosomal systems for physical characteristic investigation

	Formula no.	Niosomal concentrations (mM)	Types and concentrations of dispersants
Niosomal concentrations and various dispersants	1	5	Distilled water
	2	10	Distilled water
	3	20	Distilled water
	4	5	20 mM phosphate buffer (pH 7.0)
	5	10	20 mM phosphate buffer (pH 7.0)
	6	20	20 mM phosphate buffer (pH 7.0)
Phosphate buffer concentrations	7	5	5 mM phosphate buffer (pH 7.0)
	8	5	10 mM phosphate buffer (pH 7.0)
	9	5	20 mM phosphate buffer (pH 7.0)
	10	5	30 mM phosphate buffer (pH 7.0)

Note : For ethanol elastic niosomes, the dispersants were added with 20% v/v ethanol

1.1.3 Measurement of deformability index (DI)

Elasticity of the vesicular membrane is an important and unique parameter for deformable vesicles. Elasticity of niosomes was determined by the extrusion method previously described (Jain et al., 2007; Manosroi et al., 2008). Briefly, the niosomal dispersion was extruded through a polycarbonate membrane filter with the pore size of 50 nm (Millipore, USA) and the constant pressure (2.5 bar). After the 10-min extrusion, the

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vesicular sizes and the weight of the niosomal dispersion was measured. The elasticity of the vesicles was expressed in the terms of deformability index according to the following equation (Bergh et al., 2001):

$$\text{Deformability index (DI)} = j\,(rv/rp)^2$$

j was the weight of the niosomal dispersion, which was extruded in 10 min through a polycarbonate filter (50 nm pore size). rv was the size of the vesicle after extrusion and rp was the pore size of the filter membrane.

1.2 Effects of dispersants on the maximum loading and entrapment efficiency of calcitonin in elastic niosomes

Calcitonin at various concentrations (0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mg/ml) was loaded in non-elastic and elastic (20%v/v ethanol) niosomes. The maximum loading of calcitonin in niosomes was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel documentation (Tasset et al., 1995). Briefly, niosomes dispersed in electrophoresis loading-buffer were loaded on the gel consisting of 20%w/v separating gel and 4%w/v stacking gel. Electrophoresis was performed at the constant 80 mv voltage until the bromophenol dye front reached the bottom of the gel. The gel was stained with coomassie blue G-250 solution for 30 min and put in the destaining solution to visualize the calcitonin bands. Electrophoretic data was documented by a gel documentation system and analysed by Quantity 1-D analysis software. The maximum loading of calcitonin in niosomes was determined from the amount of the loaded calcitonin that the free calcitonin band was first seen in the gel.

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The area and intensity of the free calcitonin bands were determined and the calcitonin concentrations were calculated from the standard curve (Liu et al., 2007c). The entrapment efficiency was expressed as the percentages of the loaded calcitonin in comparing to the total drug according to the following equation:

$$\% \text{ entrapment efficiency} = (C_e/C_t) \times 100$$

C_e was the concentration of calcitonin loaded in niosomes and C_t was the total concentration of calcitonin initially loaded in the niosomal dispersion.

1.3 Effects of phosphate buffer concentrations on physical characteristics of elastic niosomes

The non-elastic and elastic niosomes were prepared with the same method as in section 1.1.1. Various concentrations (5, 10, 20 and 30 mM) of phosphate buffer solution (pH 7.0) were used to hydrate the film. The phosphate buffer solution was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate. The pH value was measured by a pH meter. Physical characteristics and deformability index of niosomes were investigated by the same methods as in sections 1.1.2 and 1.1.3, respectively.

1.4 Comparison of physical characteristics and cytotoxicity of elastic niosomes

1.4.1 Preparation of elastic niosomes

Niosomes were prepared with the same method as in section 1.1.1. For elastic niosomes containing ethanol, the 5 mM phosphate buffer solution at pH 7.0 containing various amount of ethanol (10, 15, 20 and 25%v/v) was used to hydrate the

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film. For elastic niosomes containing NaC and NaDC, various amounts of NaC and NaDC at 0.25, 0.5, 2.5, 5 and 10% mole were mixed with Tween 61 and cholesterol before hydration and the film was dispersed by 5 mM phosphate buffer solution (pH 7.0). [The %mole means the mole ratio in the niosomal formulation. The 5% mole NaC means 5% of the total moles of the other niosomal compositions besides NaC (Tween 61/cholesterol/NaC = 1:1:0.1).] Physical characteristics and deformability index of niosomes were investigated by the same methods as in sections 1.1.2 and 1.1.3, respectively.

1.4.2 Morphology of elastic niosomes

The shape and morphological features of niosomes were investigated by staining the niosomal dispersions with 1% methylene blue on the slides under an optical microscope. For confirmation of the lamellar structures, a drop of niosomal dispersion was applied on a 300-mesh formvar copper grid on paraffin and allowed the sample to adhere for 10 min. The remaining dispersion was removed and a drop of 2% aqueous solution of ammonium molybdate was applied for 5 min. The remaining solution was then removed, air dried and examined by a Transmission electron microscope (TEM) operated at 80 kV. The morphology and lamellarity of the niosomes were observed.

1.4.3 Entrapment efficiency of calcitonin loaded in elastic niosomes

The entrapment efficiency of salmon calcitonin loaded in elastic niosomes was determined with the same method as in section 1.2. Calcitonin loaded in each elastic niosomal group (ethanol, NaC and NaDC) from section 1.4.1 which gave the highest deformability index was used to determine the entrapment efficiency.

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1.4.4 Cytotoxicity of elastic niosomes

1.4.4.1 Cell cultures

The human skin fibroblast was obtained from Faculty of Tropical Medicine, Mahidol University in Bangkok, Thailand. The cells were maintained as adherent cells in T75 culture flasks at 37˚C in a humidified air incubator containing 5% CO_2. Dulbecco's modified Eagle's medium (DMEM) were supplemented with 10% fetal bovine serum , 100 U/ml of penicillin, and 100 mg/ml of streptomycin and used as the growth medium. For subculture, the cells were rinsed with phosphate buffer saline at pH 7.4 and finally detached with trypsin-EDTA solution (0.25%). The cells at the 25th passage were used.

1.4.4.2 Cytotoxicity assay

Cells were seeded in 96-well plates at an amount of 10,000 cells/well and allowed to attach overnight. Then, cells were exposed to various concentrations of the niosomal dispersions (0.05, 0.125, 0.25, 0.375 and 0.5 mM) and calcitonin solution (2.2, 5.5, 11, 16.5 and 22 mg/ml which were equivalent to those loaded in the niosomal dispersions) for 24 h. After incubation, the adherent cells were fixed by adding 50% w/v cold trichloroacetic acid and further incubated for 1 h at 4˚C. Then, the cells were rinsed with distilled water, air-dried and stained with 0.4% SRB in 1% glacial acetic acid for 30 min at room temperature (27±2°C). The unbound SRB was removed by washing with 1% glacial acetic acid solution for four times. After air-drying, 100 µl per well of 10 mM Tris base were added to dissolve the bound stain (Papazisis et al., 1997). After mixing, the absorbance was measured at 540 nm with a microplate reader. The untreated cells

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were used as a negative control. Cell viability (%) was calculated using the following equation (Bouquet et al., 2009; Han et al., 2009):

$$\text{Cell viability (\%)} = (\text{Absorbance}_{\text{treated cells}} / \text{Absorbance}_{\text{untreated cells}}) \times 100$$

where absorbance $_{\text{treated cells}}$ was the optical density at 540 nm of the treated cells, while absorbance $_{\text{untreated cells}}$ was the optical density at 540 nm of the untreated cells.

Part 2 : Chemical stability and transdermal absorption of elastic niosomes loaded with the model drug (calcitonin)

2.1 Physico-chemical stability of calcitonin loaded in niosomes

Calcitonin loaded niosomal dispersion was kept in bottles tightly covered with caps at 4±2, 27±2 and 45±2°C for 4 weeks. At 1, 2, 3 and 4 weeks, the physical characteristics (appearance, color, and sedimentation) of the dispersion were observed visually. The vesicular size and zeta potential values were measured by dynamic light scattering. The samples were withdrawn and the calcitonin contents remaining in the samples were analyzed by HPLC using the reversed-phase column. The 0.05% TFA in acetonitrile and 0.1% TFA in distilled water were used as mobile phases A and B, respectively delivered at 1 ml/min. HPLC was performed at ambient temperature with the gradient of solvent A/solvent B at 7:93. The gradient of the mobile phase was altered gradually from 7:93 to 100:0 of solvent A/solvent B. An amount of 20 µl of the injection volume was eluted in the column and monitored at 214 nm UV-detector. Each sample was lysed by 10% Triton X-100 and filtered through a 0.45-µm membrane filter, prior to

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injection onto the HPLC column. The peak areas were calculated and the concentrations of calcitonin were determined from the standard curve.

The degradation rate constants (k) of calcitonin were estimated from the slope of the plot between calcitonin concentrations versus times, by the least-squares fitting of various kinetic equations including zero order, first order and second order. The predicted shelf life (t_{90}) and half life (t_{50}), which were the time required when calcitonin in the formulations remained at 90 and 50%, respectively were estimated by substituting k into the following shelf life and half life equation (Manosroi et al., 2002; Florence et al., 2006):

$$\text{Shelf life } (t_{90}) = 0.11/[A_0]k_2$$

$$\text{Half life } (t_{50}) = 1/[A_0]k_2$$

where $[A_0]$ was the amounts of calcitonin (mg/ml) at initial and k_2 was the degradation rate constants of the second order kinetic equation.

2.2 Transdermal absorption by vertical Franz diffusion cells

2.2.1 Preparation of the rat skin

The male Sprague-Dawley rats (10-12 weeks, 150-200 mg) were obtained from National Laboratory Animal Centre, Mahidol University, Nakhon Pathom in Thailand. The hair on the abdominal skin was shaved off and left overnight. The rats were sacrificed and the abdominal skin was separated. The subcutaneous fat was then trimmed off by a scalpel. The skin was freshly used. The investigational protocol for all procedures has adhered to the “Principles of Laboratory Animal Care”.

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2.2.2 Sample preparation

The samples used for the transdermal study were calcitonin loaded in non-elastic niosomes, calcitonin loaded in elastic niosomes and calcitonin solution in phosphate buffer (pH 7.0). All formulations contained 2 mg/ml of calcitonin.

2.2.3 Transdermal absorption experiment

The rat skin was mounted on the receiver compartment solution of the Franz diffusion cells with the stratum corneum (SC) side facing upwards to the donor compartment. One milliliter of each sample was placed in the donor compartment and covered with paraffin film. The available diffusion area of the rat skin was 2.46 cm^2. The receiver chamber was filled with 14 ml of phosphate buffer saline (pH 7.4), controlled at 37±2 ºC and constantly stirred at 100 rpm with a small magnetic bar throughout the experiment. The diffusion cells were withdrawn at 1, 3 and 6 h. Calcitonin contents in the whole skin and the receiver compartment solution were extracted and analyzed by HPLC with the same method as in section 2.1. All experiments were done in triplicate.

2.2.4 Extraction of calcitonin from the treated samples

After the experiment, the rat skin was removed from the diffusion cells and swung twice in 100 ml of distilled water. The rinsed water was discarded. The skin was cut into small pieces and pooled in a vial containing 5 ml of distilled water. The vial was vortexed for 5 min and centrifuged at 18,000 rpm, 4°C for 10 minutes. The supernatant was collected and dehydrated using a freeze dryer. Then, the freezed-dried powder was reconstituted with 1 ml of 10% Triton X-100 and analyzed for calcitonin contents by

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HPLC. The receiver compartment solution was collected and dried using a freeze dryer and analyzed for calcitonin contents by HPLC with the same method as in section 2.1.

2.2.5 Data calculation and statistical analysis

The fluxes at the steady state, J ($mg/cm^2/h$), of the samples were calculated using the linear part of the correlation between the accumulated amounts of calcitonin, Q (mg/cm^2) that permeated the rat skin by unit area and time. Data were expressed as the mean ($\bar{x}$) of the three experiments±the standard deviation (SD) and were analyzed using ANOVA with LSD test. Statistical analysis differences yielding $p<0.05$ were considered significant.

Part 3 : Preparation and biological activities of the extracted protease enzymes

3.1 Preparation of the crude extracts containing protease enzymes

For the crude extract containing papain, the papaya latex was obtained from the peel of the unripe papaya fruits by making 1-2 mm deep longitudinal incusions on the fruit surfaces using a stainless steel knife. The exuded latex was collected into a glass container and stored at -20˚C until use.

For the crude extract containing bromelain, the stalk (central core) of the ripe pineapple fruit was separated from the fleshy fruits. The flesh of the fruit portion was then cut into small pieces and crushed with a blender. The juice was filtered through a cloth to remove the fibrous materials. The filtrate was centrifuged at 10,000 ×g for 10 min to remove insoluble materials. The obtained clear supernatant was filtered again

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through the Whatman filter paper No.5 and stored at 4˚C until use (Devakate et al., 2009).

3.2 Partial purification of the protease enzymes by precipitation

Papain was separated from the papaya latex by precipitation with the solution containing 95% ethanol, sodium chloride and saturated ammonium sulfate at the weight ratio of 1:3, 3:1 and 2.5:1, respectively (Chaiwut et al., 2007). For bromelain, it was seperated by the slow addition of 95% ethanol, cooled (4˚C) acetone and the saturated ammonium sulfate at the volume ratio of 1:1 with constant stirring (Doko et al., 1991; Adulyatham et al., 2006). The stirring was continued for 30 min to allow the equilibration between the dissolved and aggregated protein. Then, the precipitated enzymes were collected by centrifugation at 10,000 ×g for 15 min. The precipitate was lyophilized to get the dry powder. The percentage yields as well as the physicochemical properties of the extracted enzymes including solubility and chemical stability in comparing to the standard enzymes were investigated. The chemical solution including strong acid (hydrochloric acid: HCl), weak acid (acetic acid: CH_3COOH), strong base (sodium hydroxide: NaOH), weak base (ammonium hydroxide: NH_4OH), reducing agent (ferric chloride: $FeCl_3$), oxidizing agent (hydrogen peroxide: H_2O_2) and acid salt (sodium acetate: CH_3COONa) was added on a drop by drop until a color change or precipitate in the enzyme solution was observed. The enzymes were stored at 4˚C until use.

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3.3 Qualitative and quantitative analysis of the protease enzymes

3.3.1 High performance liquid chromatography (HPLC) analysis

The qualitative and quantitative analysis of the protease enzymes were performed by HPLC using the reversed-phase column. The mobile phase for papain was acetonitrile/ distilled water (7:3, v/v) containing 0.05% TFA, whereas that for bromelain was acetonitrile/distilled water (55:45, v/v) containing 0.1% TFA delivered at 1 ml/min. An amount of 20 μl of the injection volume of the enzyme in distilled water was eluted in the column and monitored at 230 and 280 nm UV-detector for papain and bromelain, respectively. All samples were filtered through the 0.45-μm membrane filter prior to injection onto the HPLC column.

3.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Various concentrations of the protease enzymes dissolved in electrophoresis loading-buffer (60 mM Tris-HCl, pH 6.8, with 10% glycerol and 2% SDS containing 25% bromophenol blue solution) were loaded on the gel consisting of the 15% w/v separating gel and 4% w/v stacking gel. The electrophoresis buffer was composed of 25 mM Tris base, 192 mM glycine and 0.1% SDS at pH 8.3. Electrophoresis was performed in the Mini-PROTEAN® 3Cell at 80 mV constant voltage until the bromophenol dye front reached the bottom of the gel. The gel was stained with coomassie blue G-250 solution for 30 min and put in the destaining solution overnight at room temperature (27±2°C) to visualize the peptide bands. Electrophoretic data were documented by a gel documentation system and analysed by Quantity 1-D analysis software. The bands of the

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enzymes which appeared in the gel were compared at the molecular weight (MW) with the protein marker and the standard enzymes. The area and intensity of all bands were calculated and the percentage purity of the enzymes was determined from the standard enzyme concentrations (Liu et al., 2007c).

3.4 Biological activities

3.4.1 Free radical scavenging assay

Free radical scavenging activities of the extracted enzymes, the standard antioxidant (vitamin C) and the standard enzymes were determined by a modified DPPH assay (Manosroi et al., 2010d). Briefly, 50 µl of five serial concentrations of the extracted enzymes or the standard enzymes (at 0.001-10 mg/ml) dissolved in distilled water and 50 µl of DPPH in ethanol solution were put into each well of a 96-well microplate. The reaction mixtures were allowed to stand for 30 min at 27±2 °C, and the absorbance was measured at 515 nm by a well reader against the blank (95%, v/v ethanol). Vitamin C (0.001-10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of free radical scavenging activity were calculated as the following:

$$\text{Free radical scavenging (\%)} = [(A-B)/A] \times 100$$

where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% of scavenging (SC_{50}) were calculated from the graph plotted between the percentages of scavenging and the sample concentrations.

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3.4.2 Lipid peroxidation inhibition activity

The antioxidant activity of the extracted enzymes was assayed by the modified Ferric-thiocyanate method (Manosroi et al., 2010d). An amount of 50 µl of five serial concentrations of the extracted enzymes and the standard protease enzymes (at 0.01-100 mg/ml) dissolved in DMSO was added to 50 µl of linoleic acid in 50% (v/v) DMSO. The reaction was initiated by the addition of 50 µl of NH_4SCN (5mM) and 50 µl of $FeCl_2$ (2 mM). The mixture was incubated at 37±2 ◦C in a 96-well microplate for 1 h. During the oxidation of linoleic acid, peroxides were formed leading to the oxidation of Fe^{2+} to Fe^{3+}. The latter ions forming a complex with thiocyanate can be detected at 490 nm. The solution without the sample was used as a negative control. Vitamin C and E (at 0.001-10 mg/ml) were used as positive controls. All determinations were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = [(A-B)/A] \times 100$$

where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% inhibition of lipid peroxidation (IPC_{50}) were calculated from the graph plotted between the percentages of lipid peroxidation inhibition and sample concentrations.

3.4.3 Human skin fibroblast cytotoxicity by the SRB assay

The human skin fibroblasts at the 7th passage from Faculty of Dentistry, Chiang Mai University in Thailand were used. Various concentrations of the enzymes from 10^{-9} to 100 µg/ml were investigated with the same method as in section 1.4.4.

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3.4.4 Gelatinolytic activity (zymography) on MMP-2

The samples (at 25 µg/ml) which showed no toxicity on human skin fibroblasts (from section 3.4.3) were tested for gelatinolytic activity of MMP-2 stimulation in comparing to concanavalin A. The cells were seeded in 6-well plates at an amount of 5×10^5 cells/well. The monolayer of cells was maintained in the culture medium without FBS for 24 h, treated with the samples and incubated for 72 h. The culture supernatants were collected to assess the gelanolytic activities of MMP-2 in the culture media.

SDS-PAGE zymography using gelatin as a substrate was performed according to the method previously described with some modifications (Kobayashi et al., 2003; Balitaan et al., 2010). Briefly, the cell culture supernatant was suspended in the loading buffer (0.125 M Tris (pH 6.8), 4% SDS and 0.04% bromophenol blue, without prior denaturation) and run on the 10% SDS polyacrylamide gel with the presence of 0.1% (w/v) gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 20 min in the renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, 2.5% Triton X-100). The gels were then incubated for 24 h at 37ºC in the developing buffer [50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100]. Gels were subsequently stained with 0.5% Coomassie brilliant blue G-250 and de-stained in 30% methanol and 10% acetic acid (v/v) at room temperature (27±2°C) to visualize the bands, and the gelanolytic activity was detected as a white band against a blue background (Kim et al., 2008). Electrophoretic data was determined by gel documentation system and analysed by the Quantity 1-D analysis software. The area (mm^2) multiplied by intensity of the

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bands on the gel was determined as the relative MMP-2 content (Carmeliet et al., 1997; Manosroi et al., 2010a). The MMP-2 stimulation in comparing to the control (the untreated systems) was calculated by the following equation:

$$\text{Relative MMP-2 stimulation} = \text{MMP-2 content}_{sample} / \text{MMP-2 content}_{control}$$

The assays were done in three independent separate experiments. The potency of MMP-2 stimulation of the samples was compared with concanavalin A.

Part 4 : Development of the elastic niosomes loaded with the extracted papain and bromelain

4.1 Preparation of blank elastic niosomes and NaC elastic niosomes loaded with the extracted papain and bromelain

The elastic niosomes were prepared with the same method as in section 1.1.1. The composition at 5 mM of Tween 61 mixed with cholesterol and using NaC as an edge activator at 1:1:0.1 molar ratio was used. The niosomes loaded with the enzymes were prepared with the extracted enzymes at various concentrations in 5 mM phosphate buffer solution (pH 7.0).

4.2 Physical characteristics of niosomes

The appearance, color and sedimentation of the niosomal dispersion were optically observed, while the size and zeta potential of the niosomal dispersions were determined by the dynamic light scattering. The morphology of the niosomes was observed by TEM.

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4.3 Measurement of deformability index (DI)

Elasticity of niosomes was determined by the extrusion measurement previously described in section 1.1.3.

4.4 The maximum loading and entrapment efficiency of the extracted papain and bromelain loaded in NaC elastic niosomes

The concentrations of the standard and extracted papain and bromelain loaded in niosomes were increased from 0.4 to 5.0 mg/ml. The maximum loading of the protease enzymes in niosomes was determined from the maximum concentration of the protease enzymes which gave no precipitation. The concentration of the extracted papain and bromelain loaded in niosomes at the maximum loading was selected to determine the entrapment efficiency of the enzyme. The enzymes loaded in niosomes was separated from the free enzymes by gel filtration, using the Sephadex G-50 (Wagh et al., 2010) as a packing material and phosphate buffer (pH 7.0) as an eluent. Eluates were collected in fractions using a fractional collector at the flow rate of 1 ml/min. The fractions containing the enzyme loaded in niosomes which were detected at 470 nm (Manosroi et al., 2008) were pooled, collected and dehydrated using a freeze dryer. Then, the freezed-dried powder was reconstituted with 1 ml of 10% Triton X-100 and analyzed for the enzyme contents by HPLC as previously described in section 3.3.1.

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4.5 Cytotoxicity on human skin fibroblasts of the NaC elastic niosomes loaded with the extracted enzymes

The human skin fibroblasts at the 7th passage from Faculty of Dentistry, Chiang Mai University in Thailand were used. Various concentrations of the free standard and extracted enzymes and those enzymes loaded in elastic niosomes from 0.00625 to 0.1 mg/ml were investigated with the same method as in section 1.4.4.

4.6 Gelatinolytic activity (zymography) on MMP-2 stimulation

The concentration of the standard and extracted enzymes which showed no toxicity on human skin fibroblasts (from section 4.5) were tested for gelatinolytic activity to determine MMP-2 stimulation in comparing to concanavalin A with the same method as in section 3.4.4.

4.7 Physico-chemical stability of the extracted protease enzymes loaded in NaC elastic niosomes

The free papain (standard and extracted) and those loaded in non-elastic and elastic niosomes were kept in the tight covered bottles and storred at 4±2, 27±2 and 45±2°C for 3 months. The physical characteristics (color and sedimentation) of the dispersions were observed visually, while the size and zeta potential were determined by the dynamic light scattering. At time intervals, the samples were withdrawn and the remaining enzyme contents were analyzed by HPLC as previously described in section

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3.3.1. Each sample was added with 10% Triton X-100 prior to injection onto the HPLC column. The remaining enzyme (%) was calculated by the following equation:

$$\text{The remaining enzyme (\%)} = \left[\frac{\text{Amounts of the total enzyme at each time interval}}{\text{Amounts of the total enzyme at initial}}\right] \times 100$$

Part 5 : Development of the PLGA nanospheres loaded with the standard papain

5.1 Preparation of the standard papain loaded in PLGA nanospheres

Four PLGA nanosphere formulations including the blank nanospheres and the standard papain (0.01 mg papain/1 mg PLGA nanospheres) loaded in PLGA nanospheres by the two methods [the emulsion solvent diffusion methods in water (ESD) and the w/o/w emulsion solvent evaporation method (ESE)] were prepared.

5.1.1 Emulsion solvent diffusion method in water (ESD)

PLGA nanospheres were prepared by the modified emulsion diffusion method in water (Kawashima et al., 1999). The schematic of the procedure was shown in **Figure 31**. Briefly, PLGA (100 mg) and standard papain (1 mg) were dissolved completely in the mixture of acetone (5 ml) and 0.01 M hydrochloric acid (0.9 ml). The resulting polymer-enzyme solution was poured into 50 ml of an aqueous PVA solution (2.0%w/v) and stirred at 400 rpm for 5 min using a propeller type agitator with three blades. The entire dispersed system was then centrifuged at 43,400 ×g for 10 min at 4°C and resuspended in distilled water to remove the free papain and PVA. This washing process was done in duplicate. The resulting dispersion was dried by a freeze dryer.

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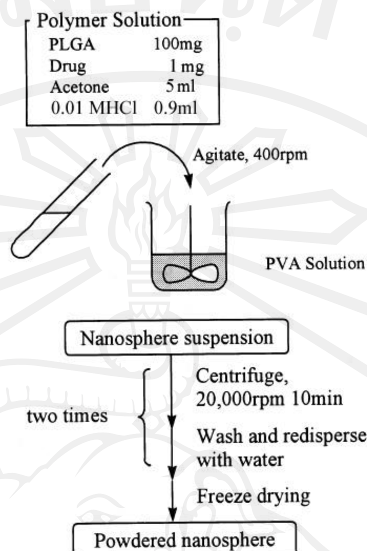

Figure 31 The procedure for the preparation of PLGA nanospheres by the emulsion solvent diffusion method in aqueous PVA solution (ESD) (Kawashima et al., 1998)

5.1.2 Water-oil-water emulsion solvent evaporation method (ESE)

The schematic diagram of the ESE procedure was shown in **Figure 32**. One hundred microliters of standard papain (1 mg) solution were emulsified in 500 µl of chloroform containing PLGA (100 mg) by sonication (duty cycle 75%, output 2) for 10 sec using a Branson Sonifier 250. The resulting primary emulsion was added to 2 ml of 10%w/v PVA and sonicated for 60 sec to form a double emulsion. The obtained emulsion was added dropwise to 18 ml of 10%w/v PVA and stirred at 400 rpm using a propeller type agitator with three blades for 3 h at room temperature (25±2°C) under evaporation to completely remove the chloroform. PLGA nanospheres loaded with the standard papain were collected by centrifugation at 43,400 ×g for 10 min at 4°C and

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resuspended in distilled water to remove the free papain and PVA. This washing process was done in duplicate. The resulting dispersion was dried by a freeze dryer.

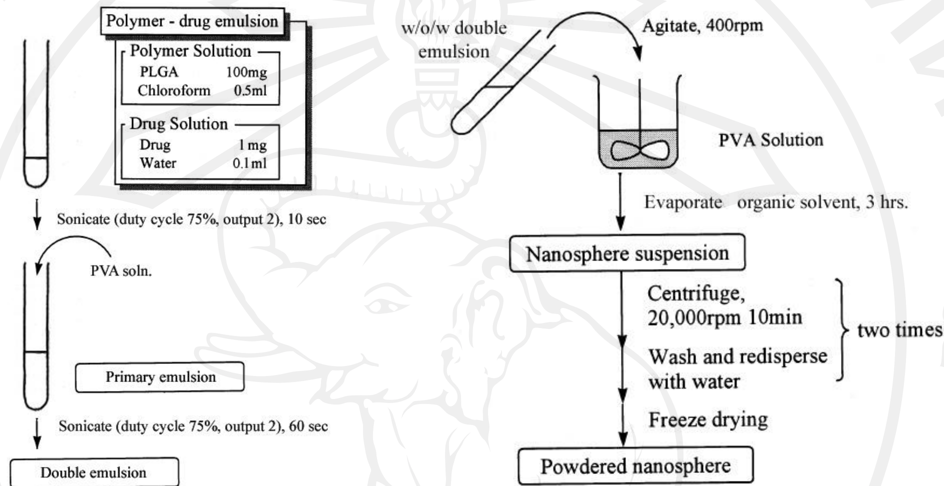

Figure 32 The procedure for the preparation of PLGA nanospheres by the water-oil-water (w/o/w) emulsion solvent evaporation method (ESE) (Tahara et al., 2009)

5.2 Physicochemical properties of the standard papain loaded in PLGA nanospheres

5.2.1 Particle size and zeta potential determination

The average particle size and zeta potential value of the PLGA nanospheres dispersed in the distilled water were determined by a laser particle size analyzer.

5.2.2 Morphology investigation

The surface topography of the freeze dried nanospheres was observed by a scanning electron microscope (SEM). The nanospheres dispersion was examined by a Transmission electron microscope (TEM).

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5.2.3 Encapsulation efficiency determination

The encapsulation efficiency of papain in PLGA nanospheres was defined as the weight ratio of papain loaded in the nanospheres to the initial loading papain amount. The freeze dried nanospheres loaded with papain were dissolved in acetonitrile, into which distilled water was added to preferentially precipitate the polymer. The papain content in the supernatant after centrifugation (43,400 ×g at 4°C for 10 min) was measured by HPLC. The encapsulation efficiency was calculated according to the following equation (Yin et al., 2006):

$$\text{Encapsulation efficiency (\%)} = \left(\frac{\text{Amounts of papain loaded in the nanospheres}}{\text{The initial amounts of papain in the systems}} \right) \times 100$$

5.3 The release profile of the standard papain from the PLGA nanospheres

Five milligrams of the nanospheres were dispersed in 5 ml of 0.2 M phosphate buffer (pH 7.0) solution and shaken horizontally at 27±2 °C and 80 strokes per min. The dispersion (1 ml) was withdrawn from the system at time intervals (0, 2, 4, 6, 8, 24 and 48 h) and centrifuged at 12,000 rpm, 4°C for 10 min. The supernatant was removed and the sediment was dissolved in 0.2 ml acetonitrile to which the distilled water was added to precipitate the polymer and to dissolve the enzyme in the aqueous mixture. The resulting suspension was centrifuged at 12,000 rpm, 4°C for 10 min to remove the precipitated polymer. The standard papain in the supernatant was determined by HPLC. In order to evaluate the release kinetics, the *in vitro* release results were fitted to various kinetic equations including zero order (% cumulative drug release vs. time), first order

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(log % cumulative drug remaining vs. time) and Higuchi matrix (% cumulative drug release vs. square root of time).

5.4 Human skin fibroblast cytotoxicity by the SRB assay

The human skin fibroblasts at the 25th passage from Faculty of Tropical Medicine, Mahidol University, Bangkok in Thailand were used. Various concentrations of PLGA nanospheres loaded with the standard papain from 0.001 to 10 µg/ml were investigated with the same method as in section 1.4.4.

5.5 Physico-chemical stability of the standard papain loaded in PLGA nanospheres

The selected preparation method of PLGA nanosphere loaded with the standard papain which gave the superior characteristics of small size, low polydispersity, high encapsulation efficiency and low toxicity was dispersed in 0.2 M phosphate buffer (pH 5.0) solution and kept in the tight covered bottles at 4±2, 25±2 and 45±2°C for 6 weeks. The physical characteristics (color and sedimentation) of the dispersions were observed visually. Particle size and zeta potential were determined by a laser particle size analyzer. At one, two, three, four and six weeks, the samples were withdrawn and the remaining enzyme contents were analyzed by HPLC. The remaining enzyme (%) was calculated by the following equation:

$$\text{The remaining enzyme (\%)} = \left[\frac{\text{Amounts of the total papain at time intervals}}{\text{Amounts of the total papain at initial}} \right] \times 100$$

* The total papain = free papain in the supernatant and papain loaded in the nanospheres

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Part 6 : Development of gel containing papain loaded in nanovesicles and nanoparticles preparation for scar treatment

6.1 Preparation of gel containing papain loaded in nanovesicles and nanoparticles

The niosomes and PLGA nanospheres loaded with papain at 1 mg/ml were incorporated into the gel base containing 8% Carbopol® 980. Briefly, the niosomes and PLGA nanospheres were dispersed in gel containing Carbopol® 980 with gentle stirring. The specified quantity of propylene glycol and concentrated paraben were added and the mixture was then neutralized with triethanolamine. The prepared gels were investigated visually for color, homogeneity and phase separation. The pH value was determined by an universal paper. The consistency of the gels was observed by a viscometer (Rotary Viscometer VR 3000. MYR Viscometers, VISCOTECH, Spain). The dynamic light scattering (Zetasizer Nano ZS British, Malvern Instruments, UK) with an angle detection at 173° was used to measure the vesicular size and zeta potential values of the nanovesicles and nanoparticles containing in the gel dispersed in phosphate buffer solution at pH 7.0.

***6.2 In vitro* rat skin transdermal absorption by vertical Franz diffusion cells**

The samples for the transdermal study through the excised rat skin were gel containing papain loaded in elastic niosomes (GEN), gel containing papain loaded in non-elastic niosomes (GNN), gel containing papain loaded in PLGA nanospheres (GPN) and gel containing free papain in phosphate buffer pH 7.0 (GS). All formulations contained 1 mg/ml of papain. One gram of each sample was used for the transdermal

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absorption experiment with the same procedure as in section 2.2. Papain contents in the whole skin and the receiver compartment solution were extracted and analyzed by HPLC.

6.3 Rabbit skin irritation test by the closed patch test

The samples for the rabbit skin irritation were papain loaded in PLGA nanospheres (PPN), papain loaded in non-elastic niosomes (PNN) and papain loaded in elastic niosomes (PEN), blank PLGA nanospheres (BPN), blank non-elastic niosomes (BNN), blank elastic niosomes (BEN), gel base (GB), gel containing papain loaded in elastic niosomes (GEN), gel containing papain loaded in non-elastic niosomes (GNN), gel containing papain loaded in PLGA nanospheres (GPN) and gel containing free papain in phosphate buffer pH 7.0 (GS).

Three male rabbits (New Zealand White, 1.5-2.5 kg) were first kept carefully following an acclimation period of 7 days to ensure their suitability for the study within a limited-access rodent facility with environmental conditions set at 25±2 °C, 60-90% RH and 12 h light/12 h dark cycle. Animals were provided *ad libitum* access to a commercial rabbit-diet and the drinking water was supplied to each cage. Back of the animals was shaved to be free of fur with an electric clipper 24 h before sample application. The shaved areas were divided into 10 sites of 2.5×2.5 cm each. An amount of 0.5 g or 0.5 ml of the samples and 20% SLS solution (the positive control) was placed on each site. The untreated site was used as a negative control. The treated sites were covered with gauze and wrapped with a non-occlusive bandage. After 24 h, the bandage and the test samples were removed and the treated sites were washed 2 times with distilled water and air dried.

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The sites were examined by an optical visualization and measured by a Mexameter® (Courage & Khazaka, Cologne, Germany) for skin edema and erythema. Scoring of erythema and edema was performed at 24, 48 and 72 h according to Draize et al. and adopted with the OECD Test Guideline 404. The Primary Irritation Index (PII) was calculated using the following equation:

$$PII = \frac{\Sigma \text{erythema grade at } 24/48/72 \text{ h} + \Sigma \text{edema grade at } 24/48/72 \text{ h}}{\text{number of animals}}$$

The irritation degree was categorized based on the PII values as negligible (PII = 0-0.4), or slight (PII = 0.5-1.9), moderate (PII = 2-4.9) or severe (PII = 5-8) irritation (Kamkaen et al., 2007). This study protocol has been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Protocol Number: 22/2554).

6.4 The physico-chemical stability of gel containing papain loaded in nanovesicles

Gels containing free papain (GS) and papain loaded in nanovesicles [gel containing papain loaded in elastic niosomes (GEN), gel containing papain loaded in non-elastic niosomes (GNN)] were kept in bottles tightly covered with caps at 4±2, 27±2 and 45±2°C for 3 months. At 1, 2 and 3 months, the physical characteristics (color, homogeneity and phase separation) of the gel formulations were observed visually. The vesicular size and zeta potential values were measured by dynamic light scattering. The pH value and specific gravity of gel were determined. The consistency of gel formulations was observed by a viscometer. At each time interval, the samples were

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withdrawn and the papain contents remaining in the samples were analyzed by the Bradford assay (Bonjoch et al., 2001).

6.5 Hypertrophic scar model in the rabbit ears

The samples used for the hypertrophic scar reduction assay were gels containing free papain (GS), papain loaded in nanovesicles [gel containing papain loaded in elastic niosomes (GEN), gel containing papain loaded in non-elastic niosomes (GNN)] and Mederma® (the commercial product containing onion extract for hypertrophic scar treatment). New Zealand White rabbits weighing between 2.5 and 3.5 kg were used in this study. Animals were anesthetized by an intraperitoneal injection of pentobarbitone sodium (60 mg/kg). Four wounds were created down to the bare cartilage on the ventral side of each rabbit ear using a 7-mm dermal biopsy punch. Epidermis, dermis, and perichondrium were thoroughly removed using a dissecting microscope. Hemostasis was then obtained by applying pressure and each wound individually covered with the polyurethane dressing (Tegaderm; 3M Health Care, St. Paul, MN). Wounds were examined every day for signs of infection as well as for the epithelialization progress on gross examination. Treatment was begun on the postoperative day 28 (Kloeters et al., 2007). Each wound was applied daily with various gel formulations from day 28 to day 56. The scars were rated based on the contour (mild: flush with surrounding skin; moderate: slightly raised or indented; severe: hypertrophic scar) and overall severity (mild, moderate, severe) using a modified visual analogue scale (VAS) (Duncan et al., 2006) and measured the height of the scar by a vernier caliper on 7, 14, 21 and 28 days

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after gel application. On the postoperative day 56, all rabbits were sacrificed and the scars were harvested (Saulis et al., 2002a). The scars were bisected through the point of maximum height of the hypertrophic scar by palpation. Individual scar samples were fixed in 4% paraformaldehyde and the tissues were then dehydrated and embedded in the paraffin using an automatic tissue processor, sectioned to 4 μm thickness with a rotary microtome, stained with hematoxylin and eosin, and then the histological morphology was examined using light microscopy. This study protocol has been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand.

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