CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Chemicals

- cholesterol(Serva Fernbiochemica, New York, USA)
- betaine hydrochloride(Sigma Chemical Co., St. Louis, MO, USA)
- N,N'-dicyclohexylcarbodiimide (DCC) (Fluka Chemicals, Gillingham, Dorset, UK)
- 4-(N,N-dimethylamino)pyridine (DMAP) (Sigma Chemical Co., St. Louis, MO, USA)
- dimethyl dioctadecyl ammonium bromide (DDAB) (Sigma Chemical Co., St. Louis, MO, USA)
- silica gel (Merck Chemicals Ltd., Poole, UK)
- dipalmitoyl phosphatidylcholine (DPPC) (Nikko Chemical Company, Tokyo, Japan)
- human insulin (Boehringer Ingelheim, Germany)
- solasodine (NPRDC, Science and Technology Research Institute (STRI), Chiang Mai University, Thailand)
- Sepharose CL-6B (Fluka Chemicals, Gillingham, Dorset, UK)
- trifluoroacetic acid (TFA) (Sigma Chemical Co., St.Louis, MO, USA)
- Other solvents (analytical grade)

2.1.2 Equipments

- analytical balance (Satorius MCl, Ac210S, Sentorius Co.,Ltd.,Gõrringen, Germany)
- fractional collector (Foxy JR, Isco, Inc., Lincoln, USA)
- ^{1}H NMR (Advance 400, Bruker)
- high perfomance liquid chromatography (HPLC, AS 1000, Thermo Finigan, USA)
- infrared spectrophotometer (FTIR-Shimadzu 8501 spectrophotometer)
- mass spectroscopy (LC/MSD 1100 series, Agilent, USA)
- lyophilizer (Christ FOC-1 Model K-40 equipment, Balzers-Pfeiffer GmbH,Asslar, Germany)
- optical microscope (type CHS, Olmpus Optical Co., Ltd., Tokyo, Japan)
- probe sonicator (Vibra CellTM, Sonics & Materials Inc., Newtown, CT, USA)95
- pH meter (Cyberscan pH 510, Euteoh Instruments, Singapore)
- rotary evaporator (R-124 Buchi, Switzerland)
- transmission electron microscope (TEM 1200S JEOL, JEOL Ltd., Tokyo, Japan)
- transmitted light differential interference contrast attachment (Model CH No. 3E0245, Olympus Optical Co. Ltd., Japan)
- Ultracentrifuge (Universal 32 R, Hettich Zentrifugen, Germany)
- zeta potential analyzer (Brookhaven Instruments Corporation, Newyork USA)

2.2 Method

2.2.1 Preparation of cationic lipids

2.2.1.1 Synthesis of cholest-5-en-3ol(3β)(trimethylammonio)acetate chloride(CTA) from cholesterol

CTA was synthesized by the DCC (N,N-dicyclohexylcarbodiimide)/DMAP (4-(N,N-dimethylamino)pyridine) coupling method with Steglich esterification. Cholesterol was dissolved in dichloromethane. Betaine hydrochloride and the solution of DMAP and DCC in dichloromethane were added to the reaction mixture. The mixture was kept at 40^{0} C under nitrogen atmosphere and the samples were withdrawn at 24, 48 and 72 hours to check with TLC in order to investigate the competition of the reaction. The reaction was quenched by adding water. The crude CTA was obtained by extraction with dichloromethane. The synthesized product was identified by melting point, infrared spectrophotometer, ^{1}H NMR, and mass spectroscopy. The synthesis process of CTA was described by the following procedures.

A. Preparation of the reagents and the equipments

Betaine hydrochloride/ cholesterol at 1:1 molar ratio was mixed. A reflux apparatus was set up, using a clean and dry condenser and an appropriate sized round-bottomed flask.

B. Synthesis of the CTA

Synthesis of CTA was performed under nitrogen atmosphere. Glass beads were added to the round-bottomed flask containing 20 ml dried dichloromethane and cholesterol (1.0 g). Betaine hydrochloride (0.4 g) was added to the mixture. The solution of 0.8 g DCC and 0.6 g DMAP in 10 ml dried dichloromethane were added to the reaction mixture was quickly reassembled. The reflux apparatus and the reaction was heated for 72 h at 40°C, while maintaining a steady reflux.

C. Reaction workup-recovery and purification of the CTA

The reaction mixture was cooled to room temperature (30 ^{0}C) and 10 ml distilled water were added in the cooled mixture and poured into a separating funnel. Then, the separating funnel was inverted several times (20 times) and the organic phase was collected. The aqueous phase was extracted with 15 ml of dichloromethane several times and separated the layers. The organic phase was collected, washed with 10 ml of distilled water. The purpose of this step was to wash away the water soluble impurities. Then, it was dried with anhydrous sodium sulphate and the solution was decanted and evaporated by a rotary evaporator. The product was collected in an appropriate sized, pre -weighed, clean and dry round-bottom flask.

D. Thin layer chromatography (TLC)

The product was identified by a TLC method. Petroleum ether and ethyl acetate (7:3, v/v) were used as a mobile phase. The spots were detected by spraying the plate with 10% sulfuric acid and then heated at 120^{0}C.

2.2.1.2 Purification of CTA

The crude CTA was purified by chromatography using a silica gel 60 column (30 cm length, 2 cm diameter) eluted with petroleum ether/ethyl acetate at 9:1 ratio with the flow rate of 1 ml/min. The fraction was collect at 5 ml per tube and checked with TLC. The pure product fractions were evaporated for the removal of the solvent using a rotary evaporator at 50 ^{0}C. The percentage yields of the CTA in the synthesized product were calculated.

2.2.1.3 Identification of the synthesis products

A. Infrared spectrophotometer (IR)

Infrared (IR) spectra of a sample in the KBr pellet were recorded from 400 to 4000 cm^{-1} using FTIR-Shimadzu 8501 spectrophotometer. Samples (0.1 mg) and 300 mg of the dried potassium bromide were mixed thoroughly by a mortar and pestle. The dried mixture was then transferred to the process for IR analysis. The obtained chromatograms were recorded and analysed for the result.

B. Nuclear magnetic resonance chromatography (NMR)

NMR spectra were performed by ^{1}H NMR Bruker Advance 400. Solubility test of the samples in $CDCl_3$ (deuterium chloroform) was performed.The samples were dissolved in 0.5 ml of the deuterated solvent and then transferred into the NMR tube by means of a glass pipette. The samples were adjusted for the depth of the solution to 45-50 mm.

C. Liquid chromatography/ mass spectrometry (LC/MS)

The test was performed using Agilent technologies LC 1100 series with Agilent technologies MSD/SL model G1946B. The spectrum was scanned between 100 and 700 m/z. The capillary voltage was $\pm$3500 V. The drying gas temperature was 350 ^{0}C. The flow rates of drying and nebulising gas (N_2) were set at 20 psi and 10 l/min, respectively.

D. Melting point

Melting point was measured using Stuart Scientific Melting point SMP1. The synthesis product was dried in a desiccator for 48 h and ground by a mortar and pestle. Then, the fine powder was filled into a capillary tube and loaded into the melting point reader. The melting points of the products were recorded.

2.2.1.4 Quantitative analysis of the synthesis products by High performance liquid chromatography (HPLC)

The synthesis products were analyzed by Thermo Finnigan HPLC system as the following:

A. Chromatographic system

A Thermo Finnigan HPLC system was equipped with Spectra system P2000 pump, C18 column, autosampler and UV detector in combination with class ChromQuest software.

B. Chromatographic conditions

Mobile phase	: acetonitrile/iso-propanol (1:1 v/v) filtered through 0.2 μm membrane filter before use
Column	: Phemomenex®, Luna 10 μm, 250 x 4.60 mm i.d.
Detector	: UV Detector at 210 nm

Flow rate : 1.0 ml/min

Injection volume : 20 μl

C. Sample preparation

A hundred milligrams of sample was weighed accurately in a 100 ml volumetric flask. Then, the mobile phase was added to dissolve and made up the volume.

D. Procedure

The instrument was adjusted as the prescribed conditions. Analysis of the sample was performed an followed the above protocol and the chromatograms were recorded. The retention time and peak area of the standard and sample were observed. The amount of the sample was calculated by comparison of the peak area to that of others.

2.2.1.5 Stability of CTA

The aliquot amount of the freshly prepared synthesized product containing CTA was filled in glass vials. The vials were kept at 4±2 ºC, room temperature (30±2 ºC) and 45±2 ºC for 3 month. The samples were quantitatively analyzed for CTA by HPLC every month.

2.2.1.6 Attempted preparation of a cationic lipid from solasodine

A cationic lipid was synthesized from solasodine and betaine hydrochloride by the DCC/DMAP coupling method with Steglich esterification. Solasodine was dissolved in dichloromethane. Betaine hydrochloride and the solution of DMAP and DCC in dichloromethane were added to the reaction mixture. The mixture was kept at 40^0 C under nitrogen atmosphere and the samples were withdrawn at 24, 48 and 72 hours to check with TLC in the order to investigate the competition of the reaction. The reaction was stop by adding water. The crude product was obtained by extraction with dichloromethane.

2.2.1.7 Preparation of a cationic lipid from cholestranol derived from cholesterol

A. Synthesis of cholestranol

Cholestranol was synthesized from cholesterol with hydrogenation reaction. Cholesterol was dissolved in dichloromethane and Pd/C was slowly added in the solution. Hydrogen gas was flow in the mixture for 8 hr**.** Then, the mixture was filtered to remove Pd/C and the filtrate was evaporated by a rotary evaporator. The product was collected and used for synthesis of cationic lipid in the next step without purification.

B. Synthesis of a cationic lipid from cholestranol

A cationic lipid was synthesized from cholestranol and betaine hydrochloride by the DCC/DMAP coupling method with Steglich esterification. Cholestranol was dissolved in dichloromethane. Betaine hydrochloride and the solution of DMAP and DCC in dichloromethane were added to the reaction mixture. The mixture was kept at 40^0 C under nitrogen atmosphere and the samples were withdrawn at 24, 48 and 72 hours to check with TLC in the order to investigate the competition of the reaction. The reaction was stop by adding water. The crude product was obtained by extraction with dichloromethane. The synthesized product was identified by melting point, infrared spectrophotometer, and ^{1}H NMR.

2.2.2 Preparations of liposomes from the synthesized cationic lipid

The purified CTA product was selected to prepare liposomes because the percentage yield of the synthesized CTA was higher than other synthesized cationic lipids.

A. Formulations of blank liposome

Liposome formulations were prepared with different molar ratios. The total concentrations of the lipids mixture were 20 mM. For liposomes, the neutral (DPPC/ Chol at 7:3 molar ratio) and cationic (DPPC/Chol/ DDAB, DPPC/Chol/CTA, DPPC/ CTA/DDAB at 7:2:1 molar ratio and DPPC/CTA at 7:3, 1:1 and 1:2 molar ratio) liposomes were prepared. All liposome formulations were prepared by a chloroform film method with sonication**.** Briefly, the lipids were dissolved in chloroform, and the solvent was removed by a rotary evaporator to get a thin film. The film was evacuated in a desiccator under reduced pressure for more than 12 h, and suspended in an aqueous solution (distilled water). The dispersion was obtained and swelled by swirling in a water bath at 50 ^{0}C for 15 min and sonicated using a microtip probe sonicator for 10 min at pulse on 3.0 and pulse off 1.0, 33% amplitude. Titanium from sonicate probe in the dispersion was removed by ultracentrifuge(Universal 32 R, Hettich Zentrifugen, Germany) at 7,000 rpm for 1 min.

B. Morphology, particle size and zeta potential determination

The morphology of liposomes was characterized by an optical microscope and a transmitted light differential interference contrast attachment (Model CH No. 3E0245, Olympus Optical Co. Ltd., Japan). The lamellarity of the liposome was observed by a TEM (80 kV, TEM1200SJEOL, JEOL Ltd., Tokyo, Japan) using negative stainning technique employing 1% (w/v) of uranyl acetate solution.

The particle size and zeta potential were measured by Zeta-sizer nanoseries. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90 ^{0}C. This measurement is based on a dynamic light scattering method. The vesicular dispersions were diluted about 30 times with distilled water before the measurement.

C. Physical stability of the blank liposomes

The liposomes were kept in transparent vials protected from light and stored at room temperature (30±2), 4±2 and 45±2 ^{0}C for 3 months. At 0,1, 2 and 3, the physical stability of dispersions was observed visually (color, odor, clarity, and precipitation). The best liposomal formulations which showed the proper physical stability were selected.

2.2.3 Entrapment of insulin in the selected liposomal formulation

A. Preparations of liposomes entrapped with insulin

The selected four liposome formulations (DPPC/ Chol at 7:3 molar ratios, DPPC/Chol/ DDAB, DPPC/Chol/CTA, DPPC/ CTA/DDAB at 7:2:1 molar ratios) were prepared by the FDEL method (Kikuchi et al., 1999). Briefly, the lipids together with cholesterol were dissolved in chloroform, and the solvent was removed by a rotary evaporator (R-124 Buchi, Switzerland) to get a thin film. The film was evacuated in a desiccator under reduced pressure for more than 12 h, and suspended in distilled water. The dispersion was obtained and swelled by swirling in a water bath at 50 ^{0}C for 15 min and sonicated for 10 min at pulse on 3.0 and pulse off 1.0, 33% amplitude, using a microtip probe sonicator (Vibra Cell™, Sonics & Materials, Inc., Newtown, CT, USA). The samples were then lyophilized by a freeze dryer (Alpha 1-2 LD model Christ, Germany). The resulting dried powder was kept at 4^{0}C until use**.** The various molar ratio of insulin to total lipid were prepared to determine the suitable ratio (Appendix B) by an aqueous solution (phosphate buffer at pH 7) of human insulin was added to various amounts of the lyophilized liposomes. The entrapment of human insulin in liposomes was performed by gently mixing with hands for 10 min and vortexing for 1 min at room temperature (30 ± 2 ^{0}C).

B. Entrapment efficiency determination of insulin in the selected liposomal formulations

The entrapped human insulin in the selected liposomes were separated from the not entrapped insulin by gel filtration using Sepharose CL 6 B (Fluka Chemicals, Gillingham, Dorset, UK) as a packing material and phosphate buffer at pH 7.0 as an eluent. Eluants were collected in fractions using a fractional collector (Foxy JR, Isco Inc., Lincoln, USA) at the flow rate of 1 ml/min. The fractions containing the entrapped insulin in liposomes which were detected at 470 nm (Miguel et al, 1997), were pooled, collected and dried with a lyophilizer (Balzers-Pfeiffer GmbH, Asslar, Germany). The fractions containing the not entrapped insulin which were detected at 210 nm, were pooled, collected and dried with a lyophilizer. The remaining residues of the liposome fractions were dissolved in 10% triton X-100(Iwanaga et al., 1997). Both pooled fractions were assayed for the insulin contents by HPLC using a reverse phase C_{18} column (Phemomenex®, Luna 10 µm, 250 x 4.60 mm i.d.) and 0.1% trifluoroacetic acid/acetonitrile(60:40 v/v) as the mobile phase with the flow rate of 1 ml/min at 210 nm(Kazunori et al, 1997). Peak areas were calculated and the concentrations of insulin were determined from the standard curves.

The percentage of entrapment efficiencies were calculated from the ratio of the amount of insulin entrapped in the liposomes (I_{in}) and the total initial amount of insulin (I_{total}) and multiplied by 100, according to the following equation:

$$\text{Entrapment efficacy (\%)} = \frac{I_{in} \times 100}{I_{total}}$$

C. Morphology, particle size and zeta potential determination of insulin entrapped in the selected liposomal formulations

The morphology of liposomes entrapped with insulin was characterized by an optical microscope. The particle size and zeta potential were measured using Zeta-sizer nanoseries. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90 ^{0}C. This measurement is based on a dynamic light scattering method. The vesicular dispersions were diluted about 30 times with distilled water before the measurement.

D. Stability study of the selected liposomes entrapped with human insulin

The liposomes entrapped with insulin were kept in transparent vials and stored at room temperature (30±2), 4±2 and 45±2 ^{0}C for 4 months. At 0, 1, 2 and 3 weeks, and 1, 2, 3, and 4 months the physical stability of the dispersions was observed visually (color, odor, clarity, precipitation and pH). At 4 months, the samples were withdrawn and human insulin contents in sample were analyzed by HPLC according to the procedure previously described in section 2.2.3 B.