CHAPTER 2

EXPERIMENTAL

2.1 Materials and equipments

2.1.1 Chemicals

- Boric acid (Fluka AG, Buchs SG, Switzerland)
- Chloroform (Analytical reagent, Lab-scan Ltd., Ireland)
- Cholesterol (Sigma Chemical Co., Louis, USA.)
- Dicetyl phosphate (Sigma Chemical Co., Louis, USA.)
- Hydrogenated soya phosphatidylcholine (Emulmetik 950®) Lot no. 179716, Lucus Meyer. JJ-Degussa (T) Ltd., Bangkok.)
- Molecular porous membrane tubing molecular weight cut off 12,000-14,000 (Spectrum®, Houston, Texas, US & Canada)
- Potassium dihydrogen orthophosphate (BDH Laboratory Supplies, England)
- Stearylamine (Sigma Chemical Co., Louis, USA.)
- Tranexamic acid (Asahi chemical industry Co., Ltd., Japan)
- 2,4,6 Trinitrobenzosulfonic acid (Sigma Chemical Co., Louis, USA.)
- Triton-X 100 (BDH Ltd., Poole, England)

2.1.2 Equipments

- Analytical balance (Sartorius MC1, AC210S, Satorius Co., Ltd., Germany)
- Differential Scanning Calorimeter (Perkin Elmer DSC7, Perkin Elmer, Ltd.,USA)
- Freeze Dryer (Model Lioalfa 10, Telstar, Spain)

- HORIBA pH-Meter (HORIBA Ltd., Kyoto, Japan)
- Light Scattering Particle Analyzer (Mastersizer S Long Bed Ver. 2.11 Serial Number, Malvern Instruments Ltd., Malvern, UK)
- Rota Vapour (R-124 Buchi, Switzerland)
- Spectrophotometer (Milton Roy Spectronic 1001 plus, USA)
- Sonicator (Vibra cell, Sonic & Material Inc., USA.)
- Thernogravimetric Analyzer (Perkin Elmer TGA 7, Perkin Elmer, Ltd.,USA)
- Transmission Electron Microscope (TEM 840A JEOL, JEOL Ltd., Japan)
- Ultracentrifuge (Centrikron T-1180, Kontron Instruments, Italy)
- Vertical Franz Diffusion Cell Apparatus (Crown Bio Scientific, Inc., Somerville, NJ., USA)

2.2 Methods

2.2.1 Preparation

2.2.1.1 Preparation of liposomes

Nine Liposome formulations were prepared by conventional chloroform with sonification method. Their compositions are listed in Table 2.1.

All liposome preparations had a total lipid concentration of 25 mg/ml. The concentrations of TA in DI water in formulation numbers 2, 5 and 3, 6 were 5% (w/v) and 10% (w/v) respectively. Formulation number 1 and 4 were blank positive and negative liposomes respectively.

The required amount of hydrogenated soya phosphatidylcholine, cholesterol and stearylamine or dicetyl phosphate in a 500 ml round bottom flask was dissolved in 60 ml of chloroform. Chloroform was slowly removed under reduced pressure (500 psi) using a rotary evaporator at 65 ^{0}C for 90 mins until a thin dried film of lipid deposited on the inner wall of

Table 2.1 : The compositions (g) of nine liposome formulations (in 60 ml DI water)

Compositions	Formulation No.								
	1	2	3	4	5	6	7	8	9
Emulmetik 950®	1.3125	1.3125	1.3125	1.2540	1.2540	1.2540	1.2015	1.2015	1.2015
CHL	0.1875	0.1875	0.1875	0.1815	0.1815	0.1815	0.1740	0.1740	0.1740
SA	-	-	-	0.0645	0.0645	0.0645	-	-	-
DCP	-	-	-	-	-	-	0.1245	0.1245	0.1245
TA	-	3.000	6.0000	-	3.0000	6.0000	-	3.0000	6.0000
Molar ratio HSC:CHL:SA or DCP	7:2	7:2	7:2	7:2:1	7:2:1	7:2:1	7:2:1	7:2:1	7:2:1
Expected charges	None	None	None	Positive	Positive	Positive	Negative	Negative	Negative

the flask was obtained. The film was flushed with nitrogen gas for 1 min. An amount of 60 ml of 5% or 10% TA solution in DI water or DI only water was added into the film together with about 10 g of glass beads. The mixture was then swelled by swirling in a water bath at 80 ^{0}C and 200 rpm for 30 mins. Large multilamellar vesicles were produced. Small multilamellar vesicles were finally obtained by sonication for 10 mins using a probe sonicator.

2.2.1.2 Freeze drying of liposomes

An amount of 5 ml of liposome dispersion was pipetted into a 10 ml glass vial and kept at – 80 ^{0}C for 24 hrs. The glass vials were put into a freeze dryer with freezing condensor at -46 ^{0}C for 48 hrs with the prefreezing for 4 hrs at –25 ^{0}C for 90 mins, -32 ^{0}C for 90 mins and –36 ^{0}C for 60 mins.

2.2.2 Physical properties study of liposome formulations

2.2.2.1 Transmission electron microscopy (TEM)

The morphology of liposomes was observed by a transmission electron microscope. A drop of liposome dispersion was applied on a 300 mesh formvar copper grid on paraffin and left for 10 mins to allow some of the liposome to adhere on the formvar. The remaining dispersion was removed by absorbing the drop with the corner of a piece of filter paper. A drop of 2% aqueous solution of uranyl acetate was applied for 5 min. The remaining solution was removed by absorbing the liquid with the tip of a piece of filter paper and the sample was air dried. The sample was then observed with a transmission electron microscope.

2.2.2.2 Thermal analysis of liposome formulations

A. Glass transition temperature (T_g) determination

An amount of 3 to 7 mg of Emulmetik 950®, cholesterol, stearylamine, dicetyl phosphate, TA, and the freeze dried liposome formulations were placed in sample pans and

then properly sealed. All samples were scanned at the rate of 40 ^{0}C/min from 20 to 500 ^{0}C by a thermogravimetric analyzer.

B. Transition temperature (T_c) and enthalpy of transition (H) determination

Thermograms were obtained using a Perkin Elmer Differential Scanning Calorimeter with a thermal analysis data station for data analysis. An amount of 3 to 7 mg of Emulmetik 950®, cholesterol, stearylamine, dicetyl phosphate, TA, and the freeze dried liposomes were placed in sample pans and properly sealed. An equal amount of DI water was placed in the reference pan. All samples were scanned at the rate of 5 ^{0}C/min from 25 to 200 ^{0}C. Indium standard and water were used to calibrate the calorimeter.

2.2.2.3 Particle size and particle size distribution of liposomes

A small aliquot of liposome dispersion was used to measure particle size and particle size distribution by a Light Scattering Particle Analyzer. The particle size range was set at 0.05 to 800 μm, beam length at 2.40 nm, dispersant refractive index at 1.3300 and the analysis model polydisperse was used.

2.2.3 Quantitative analysis of tranexamic acid (TA)

Amounts of TA were determined by spectrophotometry. TA was first derivatived with 2,4,6 trinitrobenzosulfonic acid. The absorbance of the yellow color derivative was measured at λ of 415 nm (Atmaca, 1989).

2.2.3.1 Preparation of the standard tranexamic acid (TA) solution

About 50 mg of standard TA were dissolved in 50 ml of DI water. A sery of TA concentration of 2.0, 4.0, 6.0, 8.0 and 10.0 mg/ml was prepared from the stock solution by an appropriate dilution technique with DI water.

2.2.3.2 Preparation of reagent solution

The 1.68% (w/v) of 2,4,6 trinitrobenzosulfonic acid solution in DI water was freshly prepared and protected from light during use.

2.2.3.3 Preparation of the buffer solution

The disodium tetraborate solution (0.025 M) was adjusted to pH 10 with the 0.1 N NaOH solution. The potassium dihydrogen phosphate solution (0.1 M) was adjusted to pH 4.5 with the same solution.

2.2.3.4 Contruction of the standard calibration curve of tranexamic acid (TA)

An amount of 0.1 ml of each standard solution was transferred into a 5.0 ml volumetric flask. After the addition of 0.25 ml of borate buffer solution (pH 10) and 0.25 ml of the reagent solution into this solution, the mixture was allowed to stand at 25 ^{0}C for 30 min. Then, the solution was diluted to 5.0 ml with phosphate buffer solution (pH 4.5). The absorbance was measured at 415 nm. The standard solution without TA was used as a blank. A Calibration graph was obtained by plotting the concentrations against the absorbance values and the regression analysis was evaluated from the data.

2.2.3.5 Determination of tranexamic acid (TA) by a titration method

An amount of TA of 2.5 g was dissolved and diluted with DI water to 100.0 ml. Then, 5 ml of the solution was diluted with DI water to 50 ml. Adjusted this solution to pH 7.0 with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. An amount of 25.0 ml of formaldehyde solution, previosly adjusted to pH 7.0, and 20.0 ml of 0.1 M sodium hydroxide was added. This solution was then titrated with 0.1 M hydrochloric acid. The end point was determinied by potentiometrically. The difference between the titrations represents the amount of sodium hydroxide required. Each ml of 0.1 M sodium hydroxide was equivalent to 15.72 mg of TA (BP., 1993).

2.2.4 Determination of tranexamic acid (TA) encapsulation efficiency in liposome

The encapsulation efficiency of TA in liposomes was determined from four preparations {i.e., 7:2:1(5%TA,+), 7:2:1(10%TA,+), 7:2:1(5%TA,-), 7:2:1(10%TA,-)}. An

amount of 1.5 g of each preparation was mixed with 1.5 g of DI water and put into a ultracentrifuge tube. The mixture was centrifuged for 90 mins by an ultracentrifuge at 150,000 g, 4 ^{0}C. The supernatant was removed and the pellet containing liposomes at the bottom of the tube was collected. The pellet was dissolved in 10% triton-X 100 solution and the supernatant was diluted with DI water.

The pellet was dissolved in 5.0 ml of 10% triton-x 100 and sonicated with a sonication bath for 20 mins. Then, 0.5 ml of this solution was diluted with 10% triton- X 100 solution to 5.0 ml. This solution was used for the analysis of the amount of the entrapped TA in liposomes.

An amount of 0.1ml of the supernatant was diluted with DI water to 10.0 ml. This solution was used for the analysis of the amount of the unentrapped TA.

An amount of 0.1 ml of above sample (TA in pellet or in supernatant) was determined by spectrophotometry and the absorbance was compared with the standard curve. The percentage of TA entrapped in liposomes was calculated from the amount of TA in the pellet divided by the total amount of TA (in pellets and supernatant) and multiplied by 100 as the following equation :

$$\%TA \text{ encapsulated in liposome} = TA_{Pel} \times 100 / (TA_{Pel} + TA_{Sup})$$

where TA_{Pel} = amounts of TA in pellet

TA_{Sup} = amounts of TA in supernatant

2.2.5 Physical and chemical stability of tranexamic acid (TA) in liposome formulations

2.2.5.1 Physical stability of tranexamic acid (TA) in liposome

A. Physical appearance

Each 5.0 ml of liposome formulation was put into a vial with stopper and kept at 4 ± 1, 30 ± 1 , and 45 ± 1 ^{0}C for 90 days. The physical stability was observed and compared (the sediment, flocculation and turbidity) at 30, 60, and 90 days.

B. The amount of the remaining tranexamic acid (TA) entrapped in liposomes of various liposome formulations

Each 20.0 ml of liposome formulation was put into a vial with stopper and kept at 4 ± 1, 30 ± 1, and 45 ± 1 ^{0}C. The sample was sampling at different time intervals of 0, 14, 30, 60 and 90 days for the determinded of percentages of TA remaining in liposomes. An amount of 1.5 g of each preparation was mixed with 1.5 g of DI water and put into a ultracentrifuge tube. The mixture was centrifuged for 90 mins by an ultracentrifuge at 150,000 g, 4 ^{0}C. The supernatant was removed and the pellet containing the liposomes at the bottom of the tube was collected.

The pellet was dissolved in 5.0 ml of 10% triton-X100 solution sonicated with a sonication bath for 20 mins. Then, 0.5 ml of this solution was diluted with 10% triton- X100 solution to 5.0 ml. This solution was used for the analysis of the amount of the remaining TA in liposomes the same as in 2.2.7.

An amount of 0.1 ml of supernatant was diluted with DI water to 10.0 ml. This solution was used for the analysis of the amount of the unentrapped TA the same as in 2.2.7.

2.2.5.2 Chemical stability of tranexamic acid (TA) in liposome formulations

An amount of 0.25 g of each liposome formulations was dissolved in 12.25 ml of 10% triton- X100 solution and sonicated with a sonication bath for 20 mins. Then, 3.0 ml of this solution was diluted with 10% triton- X100 solution to 10.0 ml. This solution was used for the analysis of the total amount of TA in liposomes by spectrophotometry method the same as mentioned above.

2.2.6 The release study of tranexamic acid (TA) from liposomes

The release study of TA from liposomes was performed by a vertical Franz diffusion cell apparatus. Molecular porous membrane tubing with the molecular weight cut off of 12,000-14,000 was used. The cells were jacketed at 37 ± 1 ^{0}C. The membrane was prepared by soaking overnight in DI water. An amount of 3.0 g of each liposome formulation was placed in an ultracentrifuge tube and centrifuged at 150,000 g, 4 ^{0}C for 90 mins. The supernatant was removed. The pellet containing the liposomes at the bottom of the tube

was collected. The pellet was resuspended in 6.0 ml of DI water. The 2.0 ml dispersion liposome was put into the donor cell of the apparatus. An amount of 12.0 ml of the DI water was put into the receiver chamber. At each time interval of 15, 30 mins, 1, 2, 4, 6, 8 and 24 hrs, 0.5 ml of the receiver media was withdrawn. After each sampling, the receiver media was replaced by the same withdrawal amount. The sample was then assayed for TA contents by the same procedure mention above.