

PREPARATION, OPTIMIZATION, CHARACTERIZATION OF LIPOSOMES CONTAINING SERRATIOPEPTIDASE FOR ORAL DELIVERY

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ABSTRACT

The liposome prepared by ethanol injection method by using Phosphatidylcholine and cholesterol at 10:10 ratio shown good % Entrapment efficiency and *In-vitro* drug release study. Optimized formulations were subjected to stability studies at two different temperatures for up to 45 day. No significant changes in drug entrapment efficiency and mean particle size were observed during the course of stability study for formulations stored at 4-8°C but there was a significant decrease in drug entrapment efficiency for liposome stored at room temperature after 15, 30 and 45 days and the mean particle size were increased at room temperature after 15, 30 and 45 days.

KEYWORDS Serratiopeptidase, liposomes, phosphatidylcholine, Cholesterol, stability studies.

INTRODUCTION

A **liposome** is a tiny bubble (vesicle), made out of the same material as a cell membrane. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases.

Liposomes were first described by British haematologist Dr Alec D Bangham FRS in 1961 (published 1964), at the Babraham Institute, in Cambridge. They were discovered when Bangham and R. W. Horne were testing the institute's new electron microscope by adding negative stain to dry phospholipids. The resemblance to the plasmalemma was obvious, and the microscope pictures served as the first real evidence for the cell membrane being a bilayer lipid structure.

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body. Structurally, liposomes are concentric blebder vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer. Membranes are usually made of phospholipids, which are molecules that have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water.¹

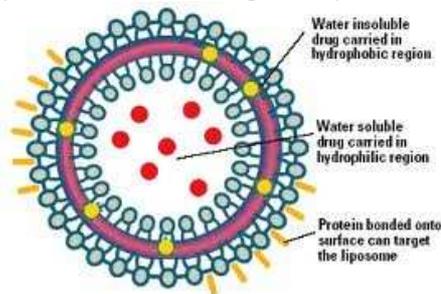


Fig. - 1: Scheme of a liposome formed by phospholipids in an aqueous solution.

In nature, phospholipids are found in stable membranes composed of two layers (a bilayer). In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment, and another layer of heads faces inside the cell, attracted by the water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bilayer.¹

When membrane phospholipids are disrupted, they can reassemble themselves into tiny spheres, smaller than a normal cell, either as bilayers or monolayers. The bilayer structures are liposomes. The monolayer structures are called micelles.

The lipids in the plasma membrane are chiefly phospholipids like phosphatidylethanolamine and phosphatidylcholine. Phospholipids are amphiphilic with the hydrocarbon tail of the molecule being hydrophobic; its polar head hydrophilic. As the plasma membrane faces watery solutions on both sides, its phospholipids accommodate this by forming a phospholipid bilayer with the hydrophobic tails facing each other.

Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine), or of pure surfactant components like DOPE (dioleoylphosphatidylethanolamine). Liposomes, usually but not by definition, contain a core of aqueous solution; lipid spheres that contain no aqueous material are called micelles, however, reverse micelles can be made to encompass an aqueous environment.²

ADVANTAGES

Some of the advantages of liposome are as follows:

- Provides selective passive targeting to tumor tissues (Liposomal doxorubicin).
- Increased efficacy and therapeutic index.
- Increased stability via encapsulation.
- Reduction in toxicity of the encapsulated agents.
- Site avoidance effect.
- Improved pharmacokinetic effects (reduced elimination, increased circulation life times).
- Flexibility to couple with site specific ligands to achieve active targeting.¹

MATERIAL AND METHODS

OPTIMIZATION OF PROCESS VARIABLES

Preparation of Liposomes

Melt Method³

Table 01: Preparation of Liposome by Melt method

Ingredients (Cholesterol: phosphatidyl)	A1 (2:1) (mg)	A2 (3:1)	A3 (4:1)	A4 (2:1)	A5 (3:1)	A6 (4:1)
Serratiopeptidase	25 mg	25 mg	25 mg	25 mg	25 mg	25 mg
Soya lecithin	200 mg	200 mg	200 mg	-	-	-
Egg phosphatidylcholine	-	-	-	200 mg	200 mg	200 mg
Cholesterol	400 mg	600 mg	800 mg	400 mg	600 mg	800 mg
HPMC K4M	25 mg	25 mg	25 mg	25 mg	25 mg	25 mg
Saline phosphate buffer (pH 7.4)	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml

Ethanol injection method⁴**Table 02: Preparation of Liposome by Ethanol injection method**

Ingredients (Phosphatidylcholine: cholesterol) ratio	A ₇ (p:c/10:3)	A ₈ (p:c/10:5)	A ₉ (p:c/10:7)	A ₁₀ (p:c/10:9)	A ₁₁ (p:c/10:10)
Serratiopeptidase	10 mg/ml	10 mg/ml	10 mg/ml	10 mg/ml	10 mg/ml
Egg Phosphatidylcholine	1000mg	1000mg	1000mg	1000mg	1000mg
Cholesterol	300mg	500mg	700mg	900mg	1000mg
Methanol:Ethanol (1:4)	10ml	10ml	10ml	10ml	10ml
HPMC K4M	25 mg	25 mg	25 mg	25 mg	25 mg
Saline Phosphate buffer (pH 7.4)	100ml	100ml	100ml	100ml	100ml

Thin Layer Evaporation Method (TLE)⁵**Table 03: Preparation of Liposome by TLE**

Ingredients (Phosphatidylcholine: cholesterol) Ratio	A ₁₂ (p:c/1:1)	A ₁₃ (p:c/2:1)	A ₁₄ (p:c/3:1)	A ₁₅ (p:c/1:1)	A ₁₆ (p:c/2:1)	A ₁₇ (p:c/3:1)
Serratiopeptidase	100 µg/ml					
Soya lecithin	250 mg	500 mg	750 mg	-	-	-
EggPhosphatidylcholine	-	-	-	250 mg	500 mg	750 mg
Cholesterol	250 mg					
Chloroform : Methanol (9:1 ratio)	20 ml					
HPMC K4M	25 mg					
Saline Phosphate buffer (pH7.4)	100 ml					

Evaluation of prepared liposomes**Size distribution profile^{6,7}**

The particle size and particle size distribution were determined using Zetasizer

Drug entrapment studies^{6,7}

Separation of untrapped drug from the prepared liposomes can be carried out by mini column centrifugation method. Liposomal suspension (0.2 ml) was placed in Sephadex G-25 column (pre-saturated with empty liposomes) and centrifuged at 2000 rpm for 3 minutes. Elutes containing drug loaded liposomes have to be collected and observed under optical microscope to ensure the absence of untrapped drug particles. Appropriate amount of elute has been digested with chloroform-methanol (2:1, v/v) and the clear solution thus obtained was analyzed spectrophotometrically (U.V./Visible spectrophotometer, Shimadzu-1601, India) for the drug content estimation at a λ max of 280 nm. Liposomes prepared without drug is treated in similar manner and served as blank for the above study. Percent drug loading (PDL) for the prepared liposomes were calculated as in Eqn.

$$PDL = \frac{\text{Entrapped drug (mg)}}{\text{Total drug added (mg)}} \times 100$$

Zeta Potential^{6,7}

The zeta potential of the liposomal formulations are determined using 0.1 M KCl buffer in demineralised water at 4-C \pm 2-C, 25-C \pm 2-C/60% \pm 5% RH, or 40-C \pm 2-C/ 75% \pm 5% RH for a period of 10, 20, and 30 days (Zetasizer).

Storage - stability studies^{6,7}

The ability of vesicles to retain the drug (i.e., drug retentive behavior) has been assessed by keeping the liposomal suspensions at four different temperature conditions, i.e., 4-8°C (Refrigerator; RF), 25 \pm 2°C (Room temperature; RT), 37 \pm 2°C and 45 \pm 2°C for a period of 5 weeks. The liposomal suspensions have kept in sealed ampoules (10ml capacity) after flushing with nitrogen. Samples are withdrawn periodically and analyzed for the drug content, in the manner described under drug entrapment studies.

In vitro drug release study:

In vitro drug release study has been conducted to examine physical stability of liposomal formulation of Serratiopeptidase upon dialysis against PBS. Briefly, 2 ml of reconstituted liposomal Serratiopeptidase sample was placed into the dialysis cassette and then suspended in a temperature-

controlled jacketed flask containing phosphate-buffer saline (PBS, pH 7.4) at 37.8°C. At various time intervals, aliquot samples are withdrawn from the flask and subjected to visual inspection and drug content analysis by a stability-indicating UV Visible Spectrophotometer^{6,7}.

RESULT AND DISCUSSION**Formulation Development****Melt Method**

Six different formulations were prepared by melt method by using Phosphatidylcholine and Cholesterol at different concentration level. All the obtained formulation were failed as there were lumps in the formulation visible by naked eyes because soya lecithin and egg phosphatidylcholine are of sticky nature.

Ethanol injection method

Further, five different formulation (A₇ to A₁₁) were prepared by ethanol injection method by using Phosphatidylcholine and cholesterol at different concentration level. All the formulation were obtained successfully and evaluated for various evaluation parameters.

Thin layer evaporation technique

Six different formulations were prepared by thin layer evaporation method by using Phosphatidylcholine and cholesterol at different concentration level. All the obtained formulation was failed due to sticky nature of soya lecithin and Phosphatidylcholine. They were strongly stuck to the flask of rotary evaporation.

Characterization of liposome:**Determination of size distribution and surface charge**

Prepared liposome batches were monitored by Malvern (laser technique)

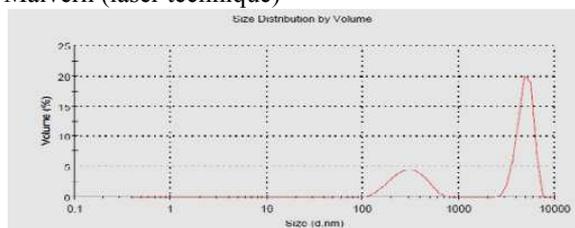


Fig. 02: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 270.3 nm for formulation A7.

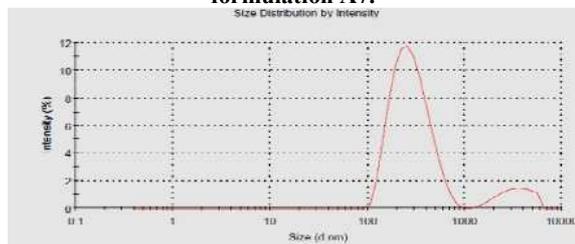


Fig. 03: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 281.1 nm for formulation A8.

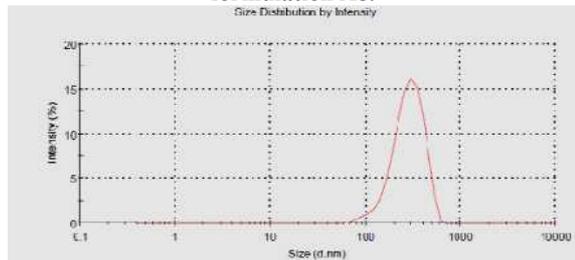


Fig. 04: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 286.7 nm for formulation A9.

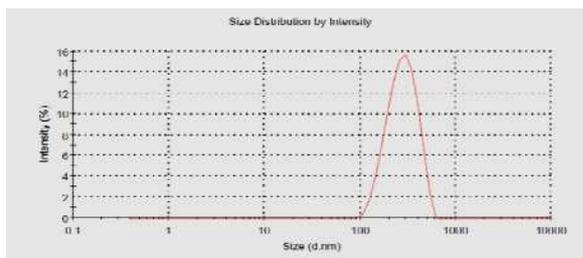


Fig. 05: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 410.4 nm for formulation A10.

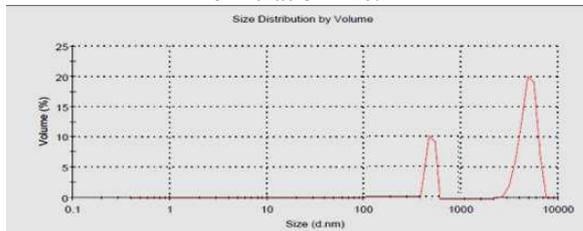


Figure No.06: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 420.5 nm for formulation A11.



Fig. 07: TEM photograph of liposome prepared by Ethanol Injection method

% Entrapment efficiency

Estimation of untrapped drug from the prepared liposome was carried out by mini column centrifugation method and % entrapment was determined for each formulation.

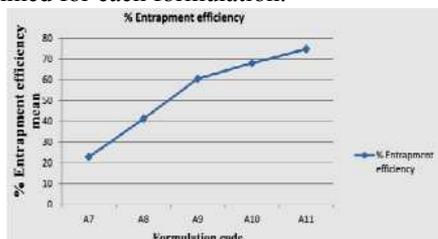


Fig. 08: % Entrapment efficiency

In the above formulation A₁₁ have 74.34 % entrapment efficiency, A₁₀ found to have 67.38 % entrapment efficiency, A₉ was found to have 62.28 % entrapment efficiency, A₈ have 40.55% entrapment efficiency and A₇ found least entrapment efficiency of 21.60 %.

Amongst all the formulation A₉, A₁₀ and A₁₁ were found to have good entrapment efficiency which is suitable for further evaluation studies. A₇ and A₈ were found to have least entrapment efficiencies of 21.60 & 40.55 % respectively.

In-vitro drug release study

Evaluation of *in vitro* drug release from encapsulated liposome was done by dialysis method. The output obtained by the dialysis method provided a correlation with the *in vitro* release.

Stability studies:

Serratiopeptidase liposomes (A₉, A₁₀, &A₁₁) were selected and their stability was evaluated at two different temperatures for up to 45 day. No significant changes in drug entrapment efficiency and mean particle size were observed during the course of stability study for formulations stored at 4-8°C but there was a significant decrease in drug entrapment efficiency for liposome stored at room

temperature after 15, 30 and 45 days and the mean particle size were increased at room temperature after 15, 30 and 45 days.

Table No. 03: Cumulative In-vitro drug release study

Time	Free drug	A9	A10	A11
30	10.54	4.39	2.49	2.5
60	21.12	9.05	8.14	6.21
90	64.42	17.55	15.44	10.52
120	85.44	28.45	24.78	15.44
150	88.77	38.4	31.25	22.98
180	89	52.41	43.65	28.22
210	89.11	64.58	54.19	32.91
240	89.11	78.45	68.18	38.48
270	89.22	82.45	77.98	47.05
300	89.22	89.45	84.11	57.14
330	89.23	92.88	90.18	67.1
360	91.31	94.1	92.13	73.15
390	94.91	94.18	93.44	83.24
420	94.92	94.8	94.91	94.92

Table no. 04: Drug entrapment efficiency of liposome formulation during Stability study

Formulation code	Freshly prepared	% Entrapment efficiency					
		At 4-8 °C			At room temperature (25 °C)		
		After 15 days	After 30 days	After 45 days	After 15 days	After 30 days	After 45 days
A9	72.55±0.41	72.23±0.2	71.56 ±1.13	72.25 ±0.88	64.19±0.86	63.36±0.62	60.45±1.04
A10	53.23±0.71	54.15±1.27	58.14 ±1.37	57.63 ±0.63	56.23±0.62	53.89±0.22	50.23 ±0.102
A11	61.58±0.53	64.32±0.78	65.58 ±0.76	60.45 ±0.86	60.78±0.63	58.78±0.62	52.95±0.68

Table No. 05 : Mean particle size of liposome formulation during stability study

Formulation code	Freshly prepared	Mean Particle size					
		At 4-8 °C			At room temperature (25 °C)		
		After 15 days	After 30 days	After 45 days	After 15 days	After 30 days	After 45 days
A9	341.26±3.04	367.98 ±4.84	367.05 ±3.34	380.14 ±6.04	389.0±6.44	412.43±4.44	447.0±6.64
A10	342.23±3.43	359.85 ±4.83	369.25 ±6.04	373.45 ±44.60	387.0±34.6	371.20±2.64	436.33±8.28
A11	346.36±3.61	340.0±3.43	352.53 ±5.34	368.32 ±8.34	366.64±6.48	390.21±6.60	418.62±4.04

CONCLUSION:

A11 formulation shown better release profile than A9 and A10.

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