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The effects of lyophilization on the stability of liposomes containing 5-FU

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Abstract

Multilamellar liposomes containing 5-fluorouracil (5-FU) were prepared by modified lipid film hydration method and were lyophilized with or without saccharose as cryoprotectant. The effect of lyophilization on the stability of liposomes was evaluated by comparing the vesicle size, encapsulation efficiency and the drug release rate before and after lyophilization/rehydration.

The process of lyophilization, without cryoprotectant, resulted in particle size increase and significant content leakage. By the addition of saccharose, the lipid bilayers become more stable and less permeable to the encapsulated drug, saccharose imparted 5-FU retention of about 80% after lyophilization/rehydration. Freeze-drying did not affect the particle size of liposomes containing saccharose as cryoprotectant.

The drug release profiles of rehydrated liposomes followed Higuchi's square root model. Also, the obtained release profiles were all biphasic: a rapid initial drug release phase (burst release of the portion of the drug that leaked out of liposomes during the lyophilization) was followed by a slower, approximately constant drug release phase (zero-order kinetics).

Keywords: Liposomes; 5-FU; Lyophilization; Cryoprotectants; Drug leakage; Stability

1. Introduction

It took only a few years following their inception as closed lipid bilayer systems (Bangham et al., 1965) for liposomes to be perceived as effective carriers for a wide variety of molecules e.g. drugs, proteins, contrast agents, genes (Lasic, 1997; Sharma and Sharma, 1997;

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Colletier et al., 2002; Slooten et al., 2000). Thus, along with the greater biophysical and biochemical understanding of liposome systems, their ability to encapsulate and deliver in vitro and in vivo a wide variety of drug molecules was thoroughly investigated. Also, knowledge of the liposome structure and physico-chemical character and their efficient or deficient performance on interaction with the biological millieu led gradually to rational design of liposome carrier systems (Kostarelos and Emfietzoglou, 1999).

Remarkable advances in the engineering of liposomes for improved delivery of its contents in vivo was the inclusion of cholesterol as part of the lipid bilayer, the use of lipids with a high-transition temperature, the inclusion of surface charge, their pattern to interact with biological molecules and cells of the RES, the techniques to achieve optimal loading of drug (Lasic et al., 1992; van Rooijen and Sanders, 1994; Semple et al., 1996).

An acceptable shelf life is a prerequisite for the successful introduction of liposomes into therapy but a disadvantage of these colloidal carriers is their chemical and physical instability in aqueous dispersions. The composition of the lipid bilayer and the aqueous phase, the amount of the external water, the bilayer–drug interaction and storage conditions are the main factors influencing liposomes stability (Sarbolouki and Toliat, 1998; Brandl, 2001).

Several approaches to monitor the stability and to stabilise liposomes on long-term storage have been developed and evaluated (Macdonald and Macdonald, 1993). To our knowledge, liposomes can successfully be lyophilized in order to achieve long-term stability. To maintain the same particle size distribution and to avoid leakage of the encapsulated drug from liposomes after the freeze-drying/rehydration cycle, a suitable cryoprotectant needs to be added (Harrigan et al., 1990; Suzuki et al., 1996; Janicki et al., 2002). Apart from the required presence of cryoprotectants, parameters as bilayer composition, freezing rate and temperature, primary and secondary freeze-drying protocol, and residual water level play a critical role (Crommelin et al., 1994; van Winden and Crommelin, 1999).

The goal of this study was to investigate the effect of lyophilization on the stability of different formulations of liposomes loaded with hydrophilic drug 5-fluorouracil (5-FU). Both size, leakage from the liposome interior and physical state of it were investigated. Also, in vitro drug release studies were performed with regard to the desired application, topically as semisolid preparation after redispersion with a suitable gel matrix, for treatment of skin deposits of the breast cancer.

2. Materials and methods

2.1. Materials

For the preparation of liposomes the following materials were used: Phospholipon 90H, gel state (PL 90H, Natterman Phospholipid, Germany); cholesterol (CHOL, Galenika, Yugoslavia); 5-fluorouracil (5-FU, Ebewe Arzneimittel, Austria) and saccharose (Merck, Germany). All other materials and solvents were of analytical grade.

2.2. Methods

2.2.1. Preparation of 5-FU loaded liposome dispersions

Liposome vesicles containing an antineoplastic agent 5-FU were prepared by lipid film hydration method (Bangham et al., 1965) with little modifications (Table 1). Briefly, required amounts of phospholipid and cholesterol (PL 90H:CHOL mass ratio = 9:1, 12:1, 15:1; marked as 1, 2 and 3 in Table 1) were dissolved in chloroform. A thin lipid film was formed on the inner side of round bottom glass container by evaporating the solvent under vacuum using a rotavapor (Devarot D3, Slovenia) at 65 °C. The film was hydrated at temperature a few degrees above the transition temperature of the lipid with different quantities of phosphate buffer pH 7.4 containing 5-FU (drug/aqueous phase mass ratio = 1:100, 1:60, 1:40; marked as *a*, *b*

Table 1
Formulations of liposomes—the initial mass ratios are given

Series Ld	*a*	*b*	*c*
Drug/aqueous phase	1:100	1:60	1:40
Lipid/aqueous phase (PL 90H-CHOL/phosphate buffer pH 7.4)	1:31.3	1:18.8	1:13.0
Phospholipid/cholesterol (PL 90H/CHOL)			
1	9:1	9:1	9:1
2	12:1	12:1	12:1
3	15:1	15:1	15:1
Drug/lipid phase	1:3.2	1:3.2	1:3.2

and *c* in Table 1). During the hydration procedure, a small glass pearls were added to stir the suspension and facilitate removal of the lipid film into the hydration medium. After 24 h, an unentraped 5-FU was removed from the liposome dispersions by ultracentrifugation (220,000 × *g*, 45 min, three times; Ultracentrifuge MLW K24D, Janetski, Germany).

Series of liposomes containing saccharose as a cryoprotectiv agent were also prepared according the method stated above. The cryoprotectant was incorporated in the internal and external aqueous phase of liposomes, so final mass ratio lipid phase: saccharose was 1:1.3.

2.2.2. Freeze-drying of liposome dispersion

Freshly prepared vesicle dispersions (series Ld) were freeze-dried in aliquots of 2 ml in 10 ml glass vials. The vials were frozen at −70 °C for 30 min. The frozen samples were placed into the drying chamber of an alpha 2–4 freeze-dryer (Martin Christ GmbH, Osterode, Germany), pre-cooled to −40 °C. Drying was performed at a pressure of 12 Pa for 24 h. After this period, a secondary drying step for 12 h at −20 °C and 1 Pa pressure was applied. The chambers were removed and the vials were filled with nitrogen gas and were closed with rubber caps and stored at 4 °C until further treatments.

The lyophilized liposomes (freeze-dried cakes) were reconstructed with phosphate buffer pH 7.4 to its original volume (series Lr) and separated from the leaked drug by ultracentrifugation.

2.2.3. Liposome characterization

The residual moisture content in the freeze-dried liposomes was measured with Mettler System, Moisture balance, PM 100, LP 16, Swiss. The result is mean of three independent determinations.

The mean particle size (geometric mean diameter d_{geo}) and size distribution analysis were carried out by use of laser diffractometry (particle size analysette D LAB/22, Fritsch, France).

Quantity of entrapped drug before and after lyophilization/rehydration was determined by HPLC. For this purpose, empty liposomes (without drug substance 5-FU) were also prepared, in the same manner and same time as liposomes with 5-FU.

Analyses were performed on Waters HPLC system, equipped with Waters 600 E Pump, sample injector Rheodyne 7725i with 20 ml loop and Waters 996 photodiode array detector. The column used was LiChrospher® 60, RP Selected B, 125 mm × 4 mm i.d., 5 μm. The mobile phase was 100% 0.02 M phosphate buffer pH 4.7. Chromatographic condition set for this method was: flow rate 1 ml/min, column temperature 20 °C, UV detection at 266 nm, injection volume 20 μm.

Approximately 4 g (accurately weighed) of the rehydrated liposomes was transferred to the volumetric flack of 50 ml, 20 ml of chloroform was added and the flack was placed in the ultrasonic bath (Branson 5510 DTH, USA) at 60 °C for 15 min. After cooling at the ambient temperature, the flack was filled with phosphate buffer pH 7.4 to the volume. The sample was centrifugated (1500 × *g*, 20 °C, 25 min; Cetrifuge Jouan MR22i) and 1 ml of the clear centrifugate was transferred to the volumetric flack and filled with phosphate buffer pH 7.4 to the volume of 10 ml. Sample solution of empty liposomes was prepared according to the same procedure.

All solutions were filtered through 0.45 μm membrane filter (Minisart RC 25; Sartorius, Goettingen, Germany), before injection. Results were calculated from linear regression of the external standard of 5-FU.

2.2.4. Drug release from reconstructed liposomes

Studies of 5-FU release from the prepared formulations were followed in vitro using dialysis through hydrophilic membrane of regenerated cellulose (Dialysis tubing D-0530, Sigma, USA). Approximately 2 g (accurately weighed) of rehydrated liposomes, as well as liposomes in which the leaked drug was removed after reconstruction of vesicles, were poured into the dialysis tubing and were dialyzed against phosphate buffer pH 7.4 as a receptor phase (25 ml, 37 °C, 100 rpm) within a period of 8 h. At predetermined time intervals, 2 ml of the receptor phase was removed and replaced with an equal volume of pre-termostated phosphate buffer pH 7.4. The released 5-FU was quantified spectrophotometrically (266 nm; Lambda 16, Perkin Elmer, USA). All dissolution tests were run in triplicate and mean values were reported.

To deduce the mechanism of the drug release from the prepared formulations, the release data's were mathematically processed.

3. Results and discussion

3.1. Characterisation of liposomes

Microscopic observations of the prepared liposomes before lyophilization (series Ld) confirmed the formation of spherical vesicles with average size of 5 μm. Particle size analysis showed no significant differences in vesicle size as a result of variations in the lipid phase composition or hydration conditions. But, the encapsulation efficiency of 5-FU was strongly affected by the formulation variable and varied in a range of 3.7–25.4%. A decrease of the CHOL amount in the lipid phase (PL 90H/CHOL mass ratio from 9:1, 12:1 to 15:1) led to an increase in the encapsulation efficiency. It is well known that by addition of cholesterol the liposome membrane became more consistent and internal aqueous volume decreased (at the loading temperature of 65 °C the liposome membrane was in the fluid-state) (Stensrud et al., 2000; Kaiser et al., 2003), so formulations with the higher amount of cholesterol in lipid phase showed smaller encapsulation efficiency of 5-FU (Fig. 1). As 5-FU does not associate with the lipid bilayer (Fresta et al., 1993), the encapsulated aqueous volume is of major importance to achieve a high-loading capacity (Elorza et al., 1993). Therefore, the encapsulation efficiency is related to the quantity of aqueous phase that is immobilized between the phospholipid bilayers and the concentration of the drug in the aqueous phase. So, the highest encapsulation efficiency was achieved in formulation Ld3c, prepared with the lipid phase of PL

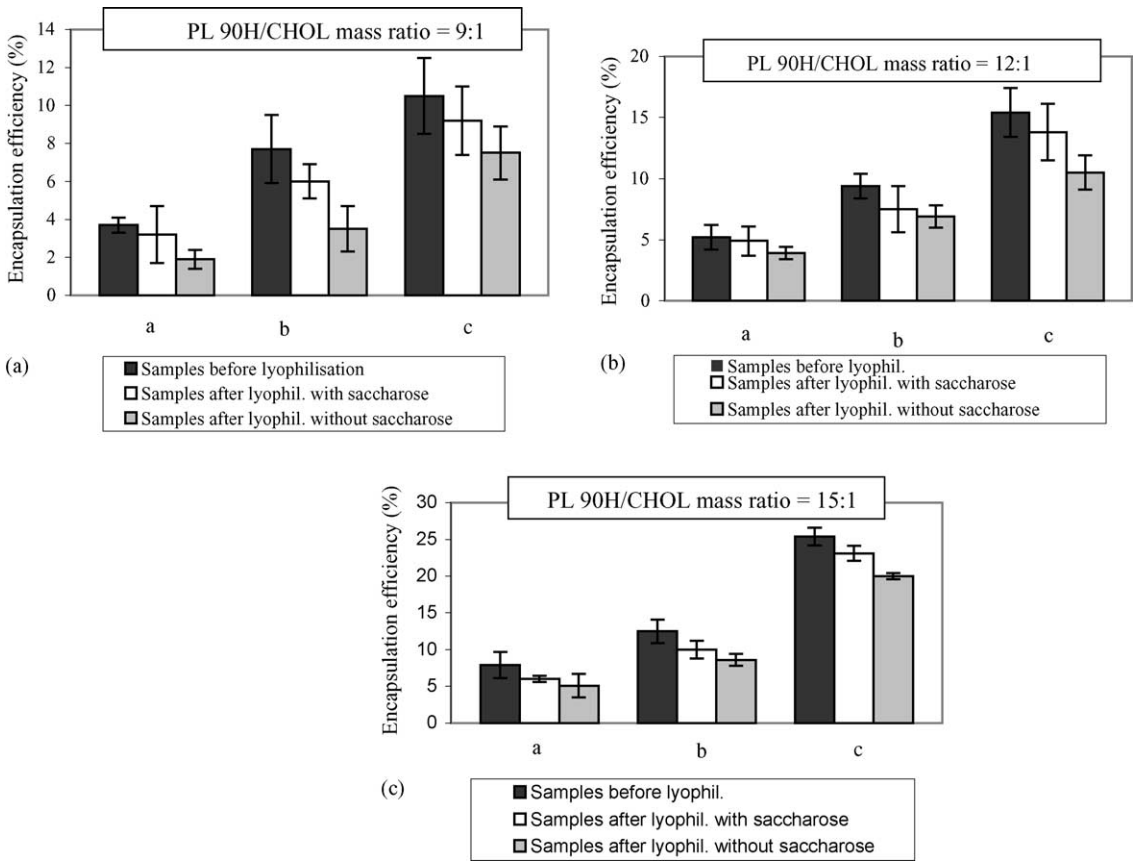

Fig. 1. Encapsulation efficiency of 5-FU into liposomes before and after lyophilization/rehydration. (a) Series Ld1 and Lr1; (b) series Ld2 and Lr2; and (c) series Ld3 and Lr3. Values are mean of five independent determinations with the error bar representing standard deviation from the mean.

90H/CHOL = 15:1 and a drug/aqueous phase mass ratio = 1:40.

Statistical analysis using one-way ANOVA (significance level, $p < 0.05$) confirmed the differences in the encapsulation efficiency due to the variations in the lipid phase composition ($F_{crit} = 3.555$, $F_{tested} = 179.99$) and hydration condition ($F_{crit} = 3.555$ and $F_{tested} = 87.37$). Interestingly, two-factorial ANOVA without replication (significance level, $p < 0.05$) showed that two experimental factors exhibit an interaction in their influence on the percentage of entrapped 5-FU in liposomes ($F_{crit} = 2.93$, $F_{tested} = 16.85$).

3.2. The effects of lyophilisation

The residual water content (w/w) in all samples of the lyophilizated liposomes was 1.03% ($n = 3$).

The process of freeze-drying did not affect the particle size of rehydrated liposomes containing saccharose as cryoprotectant, the geometric mean diameter was 5.1 ± 1 μm before lyophilization and 5.2 ± 0.4 μm after lyophilization/rehydration (Table 2). Those values are not different from each other ($p > 0.05$), although the reduction in S.D. may indicate that the heterogeneity is lower after lyophilization. In any case, it appears that there is no major change in the size of the liposomes following lyophilization. On the other side, formulations prepared without saccharose as cryoprotectant showed an increase in vesicle size after lyophilization.

The process of lyophilization was harmful for the liposome integrity also, as freezing caused a pronounced decrease in the encapsulation efficiency. Briefly, freeze-drying tended to destroy the membrane function of the phospholipid bilayer (Ozer and Talsma, 1989). Particle size increase after freeze-drying was in good agreement with the drug leakage (Stensrud et al., 2000). Sugars are well-known cryoprotectiv agents (Li et al., 2000). Their protective effect has been related to their ability to interact with the polar head groups of the phospholipids and to stabilise the membranes when the bilayer stabilising water is removed by sublimation (Engel et al., 1994; van Winden et al., 1997; Stevens and Lee, 2003.). In addition, they form an amorphous, glassy matrix during freezing and exhibit a low-molecular mobility after drying. This glass formation is considered essential to prevent damage by, e.g., fusion processes and crystal formation which lead to leakage of encapsulated drug (van Winden and Crommelin, 1999), so by addition of saccharose as cryoprotectant only a small decrease in the encapsulation efficiency was observed (Fig. 1). The drug retention was higher than 77, 80 and 76% of initially encapsulated drug for formulation Lr1, Lr2 and Lr3, respectively.

Table 2
The effect of lyophilisation on the mean particle size of liposomes

Series of liposomes	Mean geometric diameter ± S.D. (μm)[a]		
	Before lyophilisation	After lyophilisation (with cryoprotectant) Series Lr	After lyophilisation (without cryoprotectant) Series Lr
Ld1			
a	5.0 ± 0.9	5.4 ± 0.1	7.0 ± 1
b	6.0 ± 1	4.9 ± 0.1	6.0 ± 0.1
c	5.0 ± 2	5.4 ± 0.2	6.8 ± 0.9
Ld2			
a	5.0 ± 0.6	5.8 ± 0.1	7 ± 2
b	5.0 ± 1	4.7 ± 0.6	6.7 ± 0.1
c	5.0 ± 2	5.0 ± 0.3	7.1 ± 0.6
Ld3			
a	5.0 ± 1	5.0 ± 1	7.2 ± 0.6
b	5.0 ± 0.6	5.2 ± 0.3	6.3 ± 0.1
c	5.0 ± 0.9	5.0 ± 2	7.0 ± 1

[a] Each column represents the mean ± S.D. of two determinations.

3.3. Drug release from reconstructed liposomes

The release profiles of 5-FU from rehydrated liposomes (samples Lr), as a function of the lipid phase composition and hydration conditions, and corresponding formulations in which the leaked drug was removed before dialysis are presented in Fig. 2. The fast release rate of 5-FU during the initial stage (till the first hour) and consecutive slow release in the following stage was observed. The release pattern during the first hour could be related to the portion of the drug substance that leaked out of liposomes during the process of lyophilization, so the release of 5-FU could not be related only to the diffusion through the lipid bilayer, which act as a membrane barrier for encapsulated drug substance, but also to vesicle disruption during liophylisation. The release of 5-FU from rehydrated liposomes as well as from formulations in which the leaked drug was removed before dialysis was not significantly different ($p < 0.05$). Anyway, slower release rate from reconstructed liposomes in which the leaked drug was removed before dialysis correlates to the release of liposome entrapped drug.

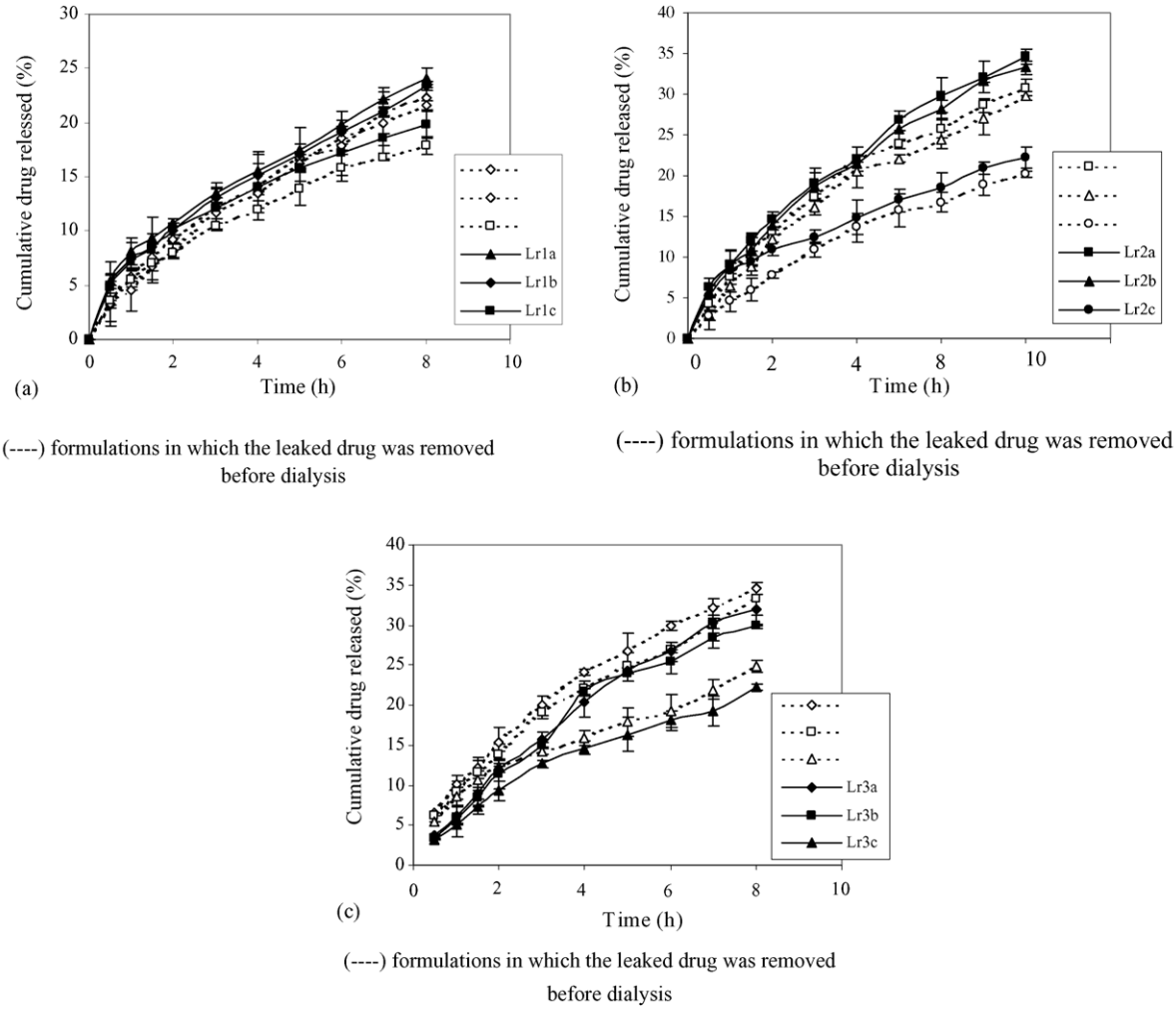

Fig. 2. The release of 5-FU from reconstructed liposomes as a function of lipid phase composition. (a) PL 90H/CH mass ratio = 9:1; (b) PL 90H/CH mass ratio = 12:1; and (c) PL 90H/CH mass ratio = 15:1. Each point represents the mean of three determinations.

Table 3
Values of kinetic parameters for the prepared liposome formulations

Formulations	Diffusion model		Zero-order kinetics[a]	
	r	k (% $h^{-1/2}$)	r	k (% h^{-1})
Lr1				
a	0.997	8.67	0.999	2.27
b	0.997	8.55	0.997	2.25
c	0.999	7.12	0.991	1.80
Lr2				
a	0.998	13.60	0.990	3.54
b	0.997	12.59	0.980	3.22
c	0.996	7.86	0.998	2.87
Lr3				
a	0.999	13.66	0.988	3.52
b	0.997	12.81	0.991	3.32
c	0.992	8.34	0.994	2.12

Each value represents the mean of three determinations. *k*: Kinetic constant of drug release; *r*: coefficient of correlation.
[a] 1–8 h.

The presence of cholesterol in the phospholipid bilayer facilitated permeation of 5-FU as incorporation of cholesterol into a gel-like membrane renders the latter more hydrophobic and less rigid. However, the addition of cholesterol decreased the amount of released 5-FU, proves that the preparation can benefit from having other factors (drug concentration, *Tc* of phospholipid, lipid/aqueous phase mass ratio), at an optimal level.

On the other side, decreasing the amount of aqueous phase bearing total drug quantity caused decrease of the drug release rate. Having in mind that liposome bilayer acts as a rate-limiting membrane barrier for encapsulated hydrophilic drug substances (Glavas-Dodov et al., 2003), higher entrapment of 5-FU into liposomes results in slower dissolution and smaller dissolution rate constant.

The 5-FU release can be fitted using a linear regression model against the square root of time (Higuchi, 1960), which hints at a diffusion-controlled process (Table 3). Also, the drug release constants obtained for the zero-order drug release kinetics (after the first hour) suggests that liposomes acted as reservoir systems for a continuous drug delivery.

4. Conclusions

Multilamellar vesicles containing an antineoplastic agent 5-FU were prepared by thin film hydration method by varying the lipid phase composition and hydration conditions. The encapsulation efficiency of 5-FU before lyophilization depended upon the PL 90H/CHOL mass ratio and a drug/aqueous phase mass ratio.

To stabilize liposomes, they were freeze-dried in presence of saccharose as cryoprotectant. By the addition of saccharose structural integrity and the permeability properties of the liposome bilayers were retained. Freeze-dried liposomes retained over 77% of the encapsulated drug upon rehydration. The mechanism of the cryoprotective effect involves binding of the sugar to the phospholipid polar groups, probably through hydrogen bonding.

This research has revealed that liposomes loaded with 5-FU and stabilized with a proper cryoprotectant under optimized and well-controlled lyophilization conditions could be suitable drug carriers in anticancer therapy.

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