

## PREPARATION OF UREA-CONTAINING STABLE PLURILAMELLAR LIPOSOMES AND STUDYING THE EFFECT OF CHOLESTEROL ON THEIR ENCAPSULATION EFFICIENCY AND RELEASE RATE

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### ABSTRACT

Liposomes have attracted much attention as a novel drug delivery system for controlled and/or targeted release of drugs. In reverse-phase evaporation, which is a well-known method of preparation for LUVs and SPLVs, the phospholipid concentration affects the preparation process, as well as characteristics of the resulting liposomes. Drug release rate from liposomes depends on permeability of the liposomal membranes. Cholesterol (CH) is quite often included in liposomal membranes to reduce their permeability to water-soluble molecules. In this study, the required concentration of the phospholipid Ovotin<sup>®</sup> 160 (O160) for the preparation of urea-containing stable plurilamellar vesicles, and the effect of different percentages of cholesterol on the encapsulation parameters and release rate of urea as a water-soluble model drug were investigated. The results show that there is a critical concentration of the phospholipid, under which the capability for the formation of a stable emulsion (in the emulsification part of the preparation process) sharply decreases. The release rate and encapsulation parameters increased when the molar ratio of cholesterol to O160 was 5% and decreased with the ratios of 50% and 100%. Therefore, in preparation of the optimum samples a balance between the encapsulation parameters as well as the release pattern should be considered carefully.

**Key words:** Liposome, SPLV, Release rate, Encapsulation efficiency, Cholesterol, Urea

### INTRODUCTION

Liposomes (phospholipid bilayer vesicles) have attracted increasing attention as a novel drug delivery system for controlled and/or targeted release of drugs. They have been used for delivery of various low and high molecular weight drugs and genes (1,2), as well as different proteins such as cytokines, which induce tumor-cell vaccination (3). They have also been suggested for topical applications (4). Another interesting application which has been suggested for liposomes, is sealing the hypoxic cells for prevention of their death (5). Different types of liposomes such as Multilamellar Large Vesicles (MLVs), Small Unilamellar Vesicles (SUVs), Large Unilamellar Vesicles (LUVs), and Stable PluriLamellar Vesicles (SPLVs) have been introduced and used for various applications. A useful method for preparation of LUVs and SPLVs is the well-known method of reverse-phase evaporation (6), in which the phospholipid concentration affects the process itself, as well as the characteristics of the final liposomal sample (6,7). Investigation of the in-vitro

release rate from liposomes is a prerequisite for understanding of their in-vivo behavior. The release rate depends on permeability of the liposomal membranes, which in turn is affected by temperature, encapsulated substance, external environment of liposomes, and composition of membrane. Cholesterol is quite often included in liposomal membranes to reduce their permeability to water-soluble molecules (8).

In this study, the required phospholipid concentration for the preparation of urea-containing SPLVs, and the effect of various percentages of cholesterol (CH) on the release rate of urea, as a water-soluble model drug, from SPLVs was investigated.

### MATERIALS AND METHODS

#### Materials:

Cholesterol (CH), Triton X-100, urea and diacetyl monoxime were from Merck Chemical Company (Darmstadt, Germany). Ovotin<sup>®</sup> 160 with egg phosphatidyl choline of approximately 60% (O160) was a gift from Faratin Co. Ltd. (Lucas Meyer representative,

Tehran, Iran). Dialysis bag (D 9777) was from Sigma Chemical Company (St. Louis, MO, U.S.A.).

*Determination of the optimum amount of phospholipid:*

To a solution of 20 mg O160 in 4ml of the organic phase (chloroform and diethylether, 1:1 v/v), was added 0.5 ml of the Phosphate buffered saline (PBS) of pH=7.4. The two-phase system were purged with nitrogen and sonicated in a bath type sonicator at 2-4 °C for 3 minutes. In another preparation, the sonication time was increased to 6 minutes in order to increase the stability of the emulsion. Due to the instability of the emulsions, several samples were prepared by increasing the amount of the phospholipid (i.e. 25, 30, 35, 40, 45, and 46 mg) under the same conditions. Results of these experiments led to the determination of the optimum amount of O160.

*Preparation of SPLVs:*

Four liposomal samples (1-4) were prepared as follow: 46 mg (66  $\mu$ mole) of O160 was dissolved in 4 ml of a 1:1 v/v mixture of diethyl ether and chloroform (sample 1). To three samples (samples 2-4) were added 1.3, 12.7, and 25.5 mg (3.3, 33 and 66  $\mu$ mole) of cholesterol respectively. Then 0.5 ml of a solution of 120 mg/ml urea in PBS (with a final pH of 7.45) was added to each. The resulting two-phase systems were purged with nitrogen and sonicated in a bath type sonicator at 2-4°C to produce stable emulsions, which were then rotary evaporated at 30-32°C, under nitrogen at a speed of 200 rpm. Final liposomal dispersions were obtained by breaking the resulting viscous gels through addition of 5ml of PBS. The preparations were then refrigerated under nitrogen for 24 hours.

*Separation of liposomes from untrapped urea:*

One ml of each of the liposomal preparations were placed inside a closed dialysis bag, and dialyzed against six 50 ml portions of PBS for ten minutes. The resulting liposomal preparations which were free of untrapped urea, were used for the following experiments.

*Determination of Encapsulation Efficiency (EF) and Captured Volume (CV) of the Liposomes:*

The dialyzed liposomal preparations obtained from the previous stage (each 1 ml) were transferred into a vessel, and the dialysis bags were washed with PBS in order to obtain the remaining amounts of the samples. After addition of 8, 10, 15, and 25 ml of Triton X-100 solution (10% v/v in PBS) to the samples 1, 2, 3, and 4 respectively for disrupting the liposomes, the entrapped urea was measured spectrophotometrically at 480 nm by diacetylmonoxime method (9). By comparing the concentration of urea in the resulting solution with

that in the aqueous solution which was used for the preparation of liposomes, the encapsulation efficiency and captured volume were calculated as percentage and L/mole of lipid, respectively. The results were then compared to each other by using ANOVA statistical test followed by Scheffe test.

*Evaluation of the release pattern:*

Franz diffusion cells containing 1 ml of dialysed samples as donor phase, 28 ml of PBS as acceptor phase, and the dialysis membrane as the semi-permeable membrane were used in these experiments, at 32° C. After 15, 30, 60, 120, 180, 240, and 300 minutes, one ml samples of the acceptor phase were taken and the urea contents were determined as mentioned above.

## RESULTS AND DISCUSSION

In this study, SPLV type of liposome was chosen because of high encapsulation efficiency, resistance to solutions with different osmotic pressures as well as biological fluids, and very low permeability which makes them a suitable candidate for sustained-release liposomal systems (10,11).

SPLVs are prepared by a kind of reverse-phase evaporation method (7). This method consists of three stages:

1. dissolution of lipids in an organic phase immiscible with water.
2. addition of the aqueous phase and emulsification of the two-phase system (by sonication).
3. removal of the organic phase.

The removal stage results in either formation of the liposomal suspension, or a viscous gel that on addition of an aqueous buffer forms the liposomal suspension. The emulsification of the two-phase system is an important process that affects the final formation of liposomes and their characteristics (10,12,13). The resulting emulsion must be reasonably stable at least for 30 minutes (6). In this study, the combination of diethylether and chloroform (1:1 v/v) was used as the organic phase in order to make the densities of the two phases similar and to prevent the creaming of the emulsified droplets. The intensity and duration of sonication are also important factors in emulsification. However, even by increasing the intensity and duration of sonication, the emulsion was still unstable. With the highest intensity and the 6 minute timing in two 3 minutes stages (which is the maximum useful time), the emulsion with 20 mg of the phospholipid showed instability only after 2 minutes. The system with 25, 30, 35, 40, 45, and 46 mg of the phospholipid remained stable for a period of 4-5, 5-7, 8-10, 12-17, 28-35, and more than 45 minutes, respectively.

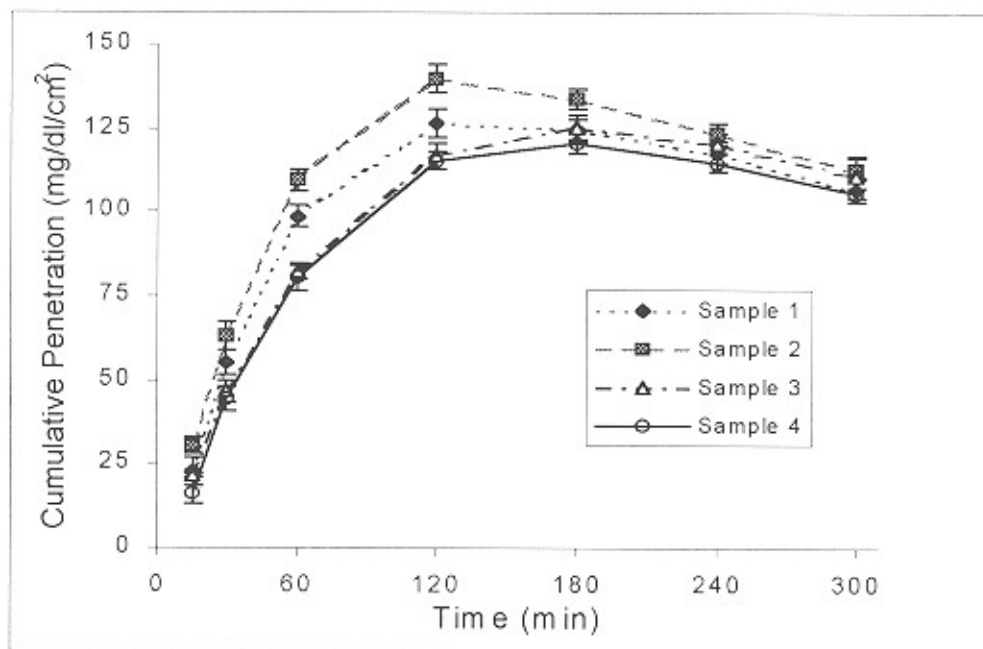
**Table 1.** Encapsulation efficiencies and captured volumes of SPLVs

SAMPLES	EF (%)	CV ( L/mole lipid)
1	51.12 ± 1.14	38.73 ± 0.85
2	55.49 ± 1.07*	42.04 ± 0.84*
3	47.70 ± 1.04**	36.14 ± 0.77**
4	46.88 ± 1.07***	35.51 ± 0.82***

Data are mean ± SD of 3 different samples. Concentrations of urea in solutions resulting from the disruption of liposomes with Triton X-100 were calculated. By comparing this concentration with that in the aqueous solution which was used for preparation of liposomes, the encapsulation efficiency and captured volume were calculated as percentage and L/mole of lipid, respectively. CH=Cholesterol and O160=Ovotin®160.

Samples: 1; O160. 2; CH:O160=5%. 3; CH:O160=50%. 4; CH:O160=100%. \* p< 0.01 different from sample 1.

\*\* p< 0.05 different from sample 1, \*\*\* p> 0.05 not significantly different from sample 3.



**Figure 1.** Drug release rate from liposomes. Data are mean ± SD of 3 different samples. Cumulative penetration is calculated as the amount of urea (per mg) in 1 dl of the samples penetrated through 1 cm<sup>2</sup> of the semi-permeable membrane to the donor phase of Franz diffusion cell. Samples: 1; O160, 2; CH:O160=5%, 3; CH:O160=50%, 4; CH:O160=100%.

Therefore, 46 mg was chosen as the optimum amount. Of course, with this concentration of the phospholipid, the removal of the organic phase led to a viscous gel, which required addition of PBS in order to form the SPLVs. The presence of cholesterol in some formulations caused easier and faster emulsification process. This is due to the higher total amount of lipids and the more rigidity of the mixed interfacial film formed around the droplets which makes the resulting emulsions more stable. In the present study, the concentrations of the entrapped material (urea) in solutions resulting from disruption of liposomes by Triton X-100, were calculated. These concentrations result from dilution of the entrapped urea into the whole samples and allow calculation of the encapsulation parameters. These parameters are shown in Table 1. In every liposomal preparation, the amounts of urea penetrated through the membrane were calculated from its concentrations in samples taken at different times from the acceptor phase. The cumulative penetration values were calculated as the amounts of urea (mg) in acceptor phase (dl) that is penetrated through  $1 \text{ cm}^2$  of the semi-permeable membrane. The plot of cumulative penetration against time is shown in Fig. 1.

Cholesterol is usually incorporated into the liposomal membranes in order to lower the fluidity of the membrane, to make them more stable in presence of biological fluids such as plasma, and to lower their permeability to water-soluble molecules. It has been suggested that the fluidity of the hydrophobic part of the liposomal bilayer is responsible for the liposomal leakage (14), and cholesterol might reduce the permeability by decreasing this fluidity. Our results (Fig. 1) show that the effect of CH on liposome permeability depends on its molar ratio to O160 in liposomal bilayer. It is speculated that molar ratios of CH to O160 in between 10 to 50% fill empty spaces between the phospholipid molecules, and anchors them stronger in the structure. This phenomenon decreases the rotational freedom of the phospholipid hydrocarbon chains and as a result, the membrane becomes more rigid and less permeable. As shown in Fig. 1, in our study, liposomes with a 50% molar ratio of CH to O160 showed a decreased permeability compared to liposomes without CH. On the other hand, as the membrane became denser, the surface area and internal volume decreased. As a result, EF and CV were reduced from  $51.12 \pm 1.14$  (%) and

$38.73 \pm 0.85$  (L/mole of lipid) in liposomes without CH to  $47.70 \pm 1.04$  (%) and  $36.14 \pm 0.77$  (L/mole of lipid) in liposomes with CH (Table 1). In 10% and lower molar ratios, CH molecules are not enough to fill all the empty spaces, and instead of making the membrane more rigid, induce a looser packing arrangement. In the present study, incorporation of CH in a 5% molar ratio to O160 into the liposomal membrane, increased the EF and CV from  $51.12 \pm 1.14$  (%) and  $38.73 \pm 0.85$  (L/mole of lipid) to  $55.49 \pm 1.07$  (%) and  $42.04 \pm 0.84$  (L/mole of lipid) respectively (Table 1). The increase in permeability is shown in Fig. 1. In these cases, an increase in surface area which is associated with a decrease in bilayer thickness (15), could be the cause of higher EF, CV, and permeability.

It is suggested that in 100% molar ratio, due to saturation, additional CH molecules destroy the order of bilayers and destabilize the membrane (16), which could increase the permeability. However, the increase in thickness of membrane compensates this effect and permeability remains unchanged in comparison with samples of 50% molar ratio (Fig. 1). On the other hand, the resultant strong "Van der Waals" attraction causes a lower internal volume, EF and CV (Table 1).

## CONCLUSION

It is concluded that there is a critical concentration of phospholipid, under which the capability for the formation of a stable emulsion (in the emulsification stage of the preparation process) is sharply reduced. Also, cholesterol, which has been normally considered as the best substance for lowering liposome permeability, is not an ideal candidate in all cases. It has a lowering effect on the encapsulation efficiency and captured volume of urea-containing SPLVs, in concentrations that cause the permeability to decrease. Therefore, the requirements with regard to the encapsulation parameters as well as the release pattern should be considered carefully to make a balance in preparation of the optimum liposomal samples.

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## REFERENCES

1. Sharma, A. and Sharma, U.S. (1997) Liposomes in drug delivery: progress and limitations. *Int. J. Pharm.* 154: 123-140.
2. Lasic, D.D. (1997) Recent developments in medical applications of liposomes: sterically stabilized liposomes in cancer therapy and gene delivery in vivo. *J. Controlled Release* 48: 203-222.
3. Slooten, M.L.V.; Storm, G.; Zoephel, A.; Kupcu, Z.; Boerman, O.; Crommelin, D.J.A.; Wagner, E. and Kircheis, R. (2000) Liposomes containing interferon-gamma as adjuvant in tumor cell vaccine. *Pharm. Res.* 17: 42-48.
4. Schmid, M.H. and Korting, H.C. (1996) Therapeutic progress with topical liposome drugs for skin disease. *Adv. Drug Del. Rev.* 18: 335-342.
5. Khaw, B.A.; Narula, J.; Vural, I. and Torchilin, V.P. (1998) Cytoskeleton-specific immunoliposomes: sealing of hypoxic cells and intracellular delivery of DNA. *Int. J. Pharm.* 162: 71-76.
6. Szoka, F. and Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci.* 75:4194-4198.
7. Gruner, S.M.; Lenk, R.P.; Janoff, A.S. and Ostro, M.J. (1985) Novel multilayered lipid vesicles, comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. *Biochemistry* 24: 2833-2842.
8. Weiner, N.; Martin, F. and Riaz, M. (1989) Liposomes as a drug delivery system. *Drug. Dev. Ind. Pharm.* 15:1523-1524.
9. Kitamura, M. and Iuchi, I. (1959) An improved diacetylmonoxime method for the determination of urea in blood and urine. *Clinica. Chimica Acta.* 4: 701-706.
10. Pidgeon, C.; McNeely, S.; Schmidt, T. and Johnson, J.E. (1987) Multilayered vesicles prepared by reverse-phase evaporation: liposomes structures and optimum solute entrapment. *Biochemistry* 26:17-29.
11. Weiner, A.L.; Carpenter-Green, S.S.; Soehngen, E.C.; Lenk, R.P. and Popescu, M.C. (1985) Liposome-collagen gel matrix: a novel sustained drug delivery system. *J. Pharm. Sci.* 74:922-925.
12. Szoka, F. and Papahadjopoulos, D. (1980) Comparative properties and methods of preparation of lipid vesicles. *Ann. Rev. Biophys. Bioeng.* 9:467-508.
13. Pidgeon, C.; Hunt, A.H. and Dittrich, K. (1986) Formation of multilayered vesicles from water/organic solvent (w/o) emulsions: Theory and practice. *Pharm. Res.* 3:23-34.
14. Muramatsu, K.; Maitani, Y.; Takayama, K. and Nagai, T. (1999) The relationship between the rigidity of the liposomal membrane and the absorption of insulin after nasal administration of liposomes modified with an enhancer containing insulin in rabbits. *Drug Dev. Ind. Pharm.* 25: 1099-1105.
15. Niven, R.W. and Schreier, H. (1990) Nebulization of liposomes: I-Effect of lipid composition. *Pharm. Res.* 7: 1127-1133.
16. Nunn, R.S. and Needham, D. (1990) Elastic deformation and failure of lipid bilayer membranes containing cholesterol. *Biophys. J.* 58: 997-1009.