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STUDY OF SOME CHARACTERISTICS OF EGG-LECITHIN LIPOSOMES PREPARED BY EXTRUSION AND FREEZE-THAWING METHOD

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تم فى هذه الدراسه تحضير الليبوزومات بطريقه التجميد والتسخين ثم الدفع لدراسه بعض الخواص لها منها محتوى الدهن لليبوزومات ، حجم الليبوزومات والنسبه المئويه لتحوصل الدليل (الكربوكسى فلورسين) داخلها. وقد تمت دراسه تأثير عدد مرات الدفع عبر غشاء عديد الكربونات والذى له حجم فتحات مختلفه هى ٨٠٠ ، ٢٠٠ و ٨٠ نانوميتر على حجم الليبوزومات المتكونه وكميه الدهن المحتوى عليها الليبوزومات وأيضا تأثير عمليه التجميد والتسخين على النسبه المئويه لتحوصل الدليل داخل الليبوزومات. وقد وجد أن كميه الدهن التى تحتوى عليها الليبوزومات لا تتغير سواء عند استخدام طريقه التجميد والتسخين أو الدفع. وأيضا توجد علاقه طرديه بين حجم فتحات الغشاء وحجم الليبوزومات المتكونه وعلاقه عكسيه بين عدد مرات الدفع وحجم الليبوزومات. ولكن بزيادة عدد مرات الدفع تتكون ليبوزومات متجانسه. وتوجد أيضا علاقه عكسيه بين عدد مرات الدفع و النسبه المئويه لتحوصل الدليل المستخدم وأن عمليه التجميد والتسخين حسنت النسبه المئويه لتحوصل الدليل داخل الليبوزومات.

Liposomes were prepared by extrusion and freeze-thawing method using carboxyfluorescein as marker. The effect of the number of extrusion, Freeze-thawing cycles on lipid content, liposome size and encapsulation efficiency was investigated. The results revealed that, there was a direct relationship between the membrane pore size and particle size of liposomes. However, there was an inverse relation between the number of extrusion cycles and size of liposomes. In the meantime, increasing the number of extrusion cycles produced homogenous liposomes. Also an inverse relation was found between the number of extrusion cycles and encapsulation efficiency of the marker. Freeze-thawing process offered high encapsulation efficiency of carboxyfluorescein inside liposomes.

INTRODUCTION

Liposomes are vesicles consisting of one or more concentric spheres of lipid layers separated by water or aqueous buffer compartment.[1] Liposomes are of great scientific, pharmaceutical and medical interest due to their ability to protect and carry hydrophilic and/or hydrophobic molecules.[2] Over the last decades liposomes have been intensively studied as carriers for dermal and transdermal application of drugs and substances used for skin care.[3,4] In the few last years, it was used very widely for ophthalmic therapy,[5,6] vaginal infections,[7] for treatment of cancer,[8] treatment of systemic fungal infections[9] and as antigens.[10] Liposome type, size, lipid composition, membrane fluidity, surface charge and method of preparation are factors that must be considered when designing liposomal carriers. Multilamellar vesicles with their large lipid compartment offer an advantage for the incorporation of hydrophilic and hydrophobic compounds.[11] Extrusion of hydrated lipid films is a common method for production of liposomes on a laboratory scale. In this study, we try to show the effect of number of passage through the extruder using polycarbonate membrane filter (different pore size) and freeze-thawing of the prepared liposomes on the size of liposomes produced, on the encapsulation efficiency of carboxyfluorescein and on lipid content of liposomes.

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EXPERIMENTAL

Materials

The following compounds were used as received from suppliers without further purification: Phosphatidyl choline from fresh egg yolk (Lipoid KG, Ludwigshafen, Germany) Carboxyfluorescein (Acros organics, New Jersey, USA). All other materials and solvents used were of analytical grade.

Methods

Liposome preparation

Liposomes constituted of phosphatidayl choline were prepared using an extrusion technique similar to that described by Olson *et al.*[12] Briefly; the lipid was dissolved in methanol. The organic solvent was slowly removed under reduced pressure on a rotary evaporator (Rotavapor R E 111, Buechi, Goepingen, Switzerland) at 30°. The produced dry thin film of lipid was hydrated at room temperature with a Hepes buffer solution (pH 7.4) containing 20 mM carboxyflourescein. The resulting liposomes were agitated well and a part of the preparation was subjected to five cycles freeze-thawing.[13,14] The vesicles were pressured in extrusion through polycarbonate membrane filters of 0.8, 0.2 and 0.08 μm pore size for one, five and eleven times using the Extruder (LiposoFast® Avestin, Ottawa, Canada).

Free carboxyfluorescein was separated from encapsulated one inside liposomes by gel chromatography using Sepharose 4B-CL.

Quantitative determination of phospholipid

Liposomal phospholipid concentration was determined according to the method of Bartlett[15] via determination of the phosphorous content of phosphatidyl choline coloriemetrically at 830 nm.

Vesicle size determination

The vesicle size distribution was measured by Photon Correlation spectroscopy (PCS) using a Malvern Zeta master, Malvern Instrument GmbH, Herrenberg, Germany. Out of the three measurements at 25° the average was taken.

Determination of trapping efficiency of carboxyfluorescein

The encapsulation percentage of carboxyfluorescein in liposomes, which prepared by extrusion and freeze-thawing method, was determined fluoriemetrically using (Luminescence spectrometer LS 50 B (Perkin Elmer, Ueberlingen, Germany) at the excitation wave length of 490 nm and the emission wave length of 520 nm and slit wides of 10 and 3.0 nm respectively.[16] The percentage of encapsulated carboxyfluorescein was calculated as:

% of encapsulated CF =

$$(A) / (A + B) \times 100 \quad \ldots\ldots\ldots\ldots (1)$$

Where:

(A): Fluorescence intensity of encapsulated carboxyfluorescein.

(B): Fluorescence intensity of free carboxyfluorescein.

RESULTS AND DISCUSSION

To study some characters of liposomes, which prepared by extrusion and freeze-thawing method, particle size and encapsulation efficiency were determined. In addition to this, the liposomes were assayed for loss of lipid after extrusion.

Phospholipid determination

The lipid content of liposomes was determined quantitatively. The amount of lipid used for preparation of liposomes was 17.0 mM. Lipid recovery was between 16.4-16.9 mM, as shown in Table 1. Lipid analysis detected no loss during liposomes preparation. This is in agreement with Berger *et al.*[17] and in contradiction to study by Jousma *et al.*[18] they reported that, there was a loss in lipid concentration when liposomes were prepared with extrusion method.

Liposome size

The prepared multilamellar vesicles by extrusion through polycarbonate membranes of 0.8, 0.2 and 0.08 μm pore size showed down-sized as illustrated in Table 1. There was no effect of freeze-thawing cycles on the size of liposomes because the extrusion of liposomes occurred after freeze-thawing cycles. Figure 1 depicted the effect of number of cycles of

Table 1: Particle size (nm), encapsulation efficiency percent and lipid content of prepared liposomes by extrusion and freeze-thawing method.

Pore size of polycarbonate membrane (μm)	Number of extrusion cycles	Particle size (nm)	Poly-dispersion	Encapsulation efficiency (%)		Lipid content (mM)	
				without FT	after FT	without FT	after FT
=====	without	1100 ± 200	0.21	55.0	60.0	16.9	16.8
0.8	One	850 ±100	0.20	49.5	55.1	16.9	16.7
	Five	800 ± 50	0.15	45.1	49.5	16.7	16.6
	Eleven	700 ± 50	0.10	40.0	44.3	16.5	16.5
0.2	One	400 ± 100	0.18	38.2	40.5	16.8	16.7
	Five	200 ± 50	0.12	31.6	36.2	16.5	16.5
	Eleven	180 ± 30	0.10	20.1	25.5	16.4	16.4
0.08	One	200 ± 50	0.18	28.5	32.5	16.6	16.5
	Five	100 ± 20	0.15	18.2	22.2	16.6	16.4
	Eleven	70 ± 10	0.10	12.2	15.1	16.5	16.5

FT: Freeze-thawing method.

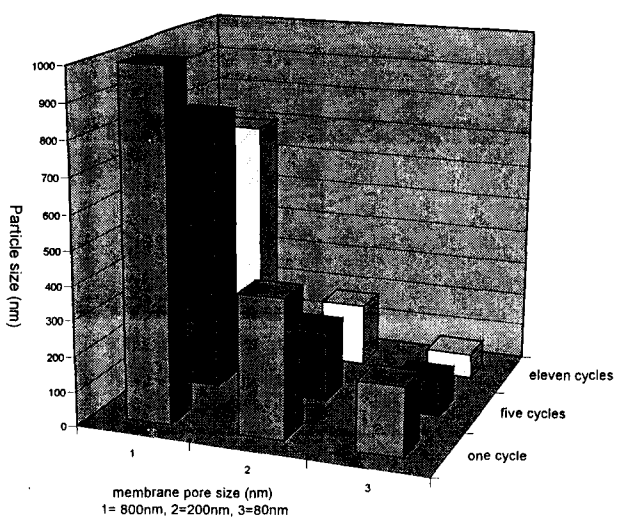

Fig. 1: Particle size of liposomes (nm) after extrusion through different polycarbonate membranes.

extrusion on the size of prepared liposomes. It appeared that, with increasing the number of cycles, the liposome size decreased and became homogenous (the polydispersion of liposomes equal 0.1, Table 1). In general, in the course of the membrane filter extrusion, a decrease in particle size with decreasing pore size was observed. Lesieur *et al.*[19] reported that, the mechanism of size reduction of liposome includes the rupturing of vesicles and spontaneous rearrangement after membrane passage resulting in the formation of smaller and less lamellar liposomes.

Percentage of encapsulation efficiency

The encapsulation efficiency percent was calculated according to equation (1). The percentage of encapsulation efficiency was shown in Table 1. It was found that, the multilamellar vesicles showed high encapsulation efficiency. The decrease of liposome size due to increase in the number of extrusion cycles could lead to decrease of encapsulation efficiency (Figures 2-4). The percentage amount of encapsulated carboxyfluorescein was markedly improved when freeze-thawing cycles were performed (Figures 2-4). This is in agreement with Castile and Taylor,[20] they reported that, when multilamellar vesicles were exposed to a series of freeze-thawing cycles, structural changes occurred and an increase in the encapsulation

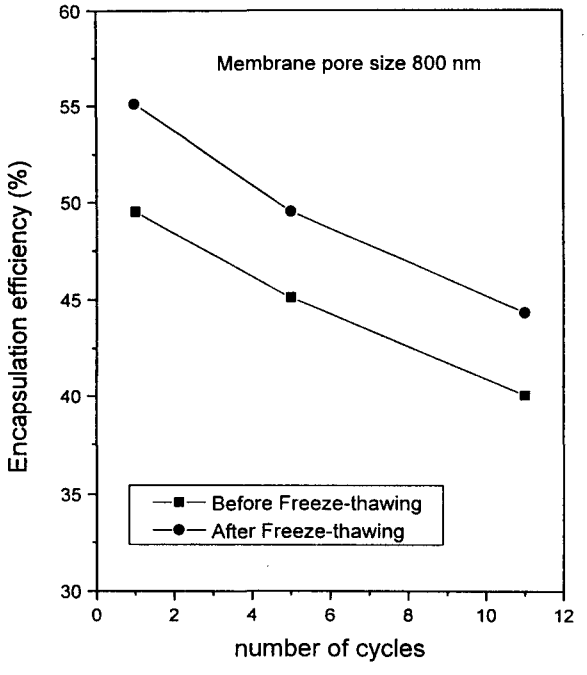

Fig. 2: Effect of extrusion cycles on the encapsulation efficiency of liposomes before and afer freeze-thawing.

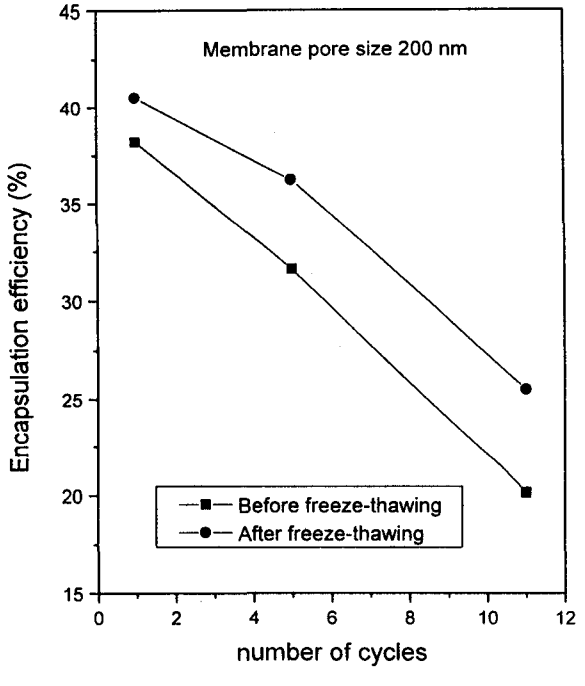

Fig. 3: Effect of extrusion cycles on the encapsulation efficiency of liposomes before and afer freeze-thawing.

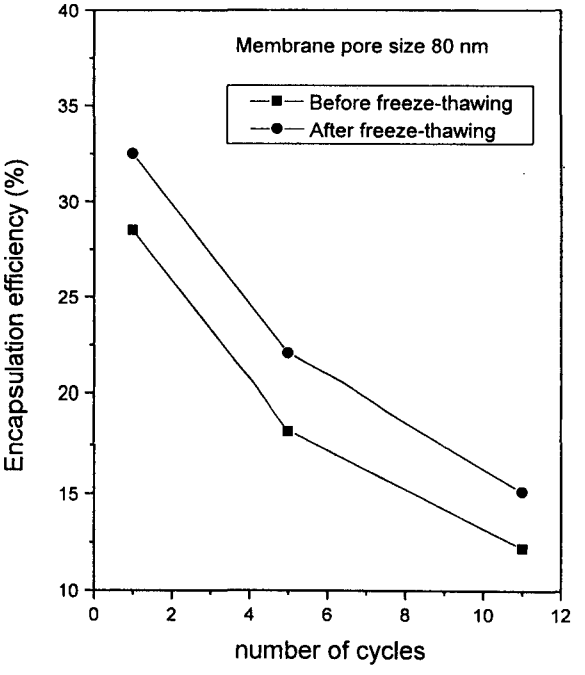

Fig. 4: Effect of extrusion cycles on the encapsulation efficiency of liposomes before and afer freeze-thawing.

efficiency resulted from improved swelling and disappearance of bilayer stacking. The trapped volume initially increased with the number of freeze-thawing cycles but reached a plateau upon continuing cycling.

CONCLUSION

The use of freeze-thawing and extrusion method for preparation of liposomes is very important to control its size. By this method, the lipid content of liposomes was not changed during preparation. Also, there was a direct relationship between the pore size of polycarbonate membrane and the size of liposomes produced. There was also an inverse relationship between the liposome size and number of extrusion cycles. Increasing the number of cycles gives homogenous liposomes. The encapsulation efficiency increased with increasing liposome size. The freeze-thawing cycles improved the encapsulation efficiency of liposomes.

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