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A NOVEL METHOD FOR PREPARING LIPOSOME WITH A HIGH CAPACITY TO ENCAPSULATE
PROTEINOUS DRUGS : FREEZE-DRYING METHOD

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A simple method for the preparation of liposome with high encapsulation efficiency was developed. A solution of L-asparaginase (A-ase) and a sonicated phospholipid were mixed together and the mixture was freeze-dried. The liposomes were regenerated by adding distilled water, followed by shaking with a Vortex mixer (FD method). Electron microscopic observation revealed that the liposomes prepared by this method have a multilamellar structure. The liposomes had approximately eight times as much encapsulating ability as those prepared by the ordinary thin film method.

KEYWORDS — liposome; preparation method; freeze-dry;
encapsulation efficiency; L-asparaginase; electron microscopy

In recent years, liposomes have been studied extensively as a drug carrier¹⁾ and a part of the work has already reached the stage of practical application.²⁾ In order to utilize liposomes for clinical use, however, they must be prepared to satisfy the following standards: (1) High degrees of drug encapsulation must be attained; (2) organic solvents and detergents must be completely removed; (3) they can be sterilized; (4) they can be made by large scale production methods; (5) the final product must be simple to use.

So far, liposomes have been prepared by various methods such as the thin film,³⁾ detergent removal,⁴⁾ reverse-phase evaporation,⁵⁾ and ether injection methods,⁶⁾ and their comparative properties and practical utilities have been widely discussed.⁷⁾ Unfortunately, most preparation methods available at present fail to meet the preceding criteria. In order to overcome these problems, we have devised and applied a novel method for preparing liposome for therapeutic use: the freeze-drying method (FD method).⁸⁾ This report describes the procedure and encapsulation efficiency of the FD method in comparison with those of the thin film method originated by Bangham et al., using L-asparaginase (A-ase) as a model drug.

The procedures for preparing liposomes by the FD method are shown in Fig. 1. Yolk phospholipid mixture (YPL) was extracted from egg yolk with 95% ethanol⁹⁾ and partially purified by an acetone extraction.^{4a)} A-ase was purchased from Kyowa Hakko Kogyo Co., Ltd. (Leunase Injection, Tokyo, Japan). YPL (0.5 g) was dispersed into 9.5 ml of 0.05 M Tris-HCl buffer (pH 8.0) using the ultrasonic disintegrator (Ultrasonic Ltd., Model 300, London, U.K.). This dispersed YPL (5%) was mixed with an equal volume of Tris buffer (pH 8.0), in which A-ase (20 IU/ml) and mannitol (5%) were dissolved. The mixture was filtered through a membrane filter (pore size

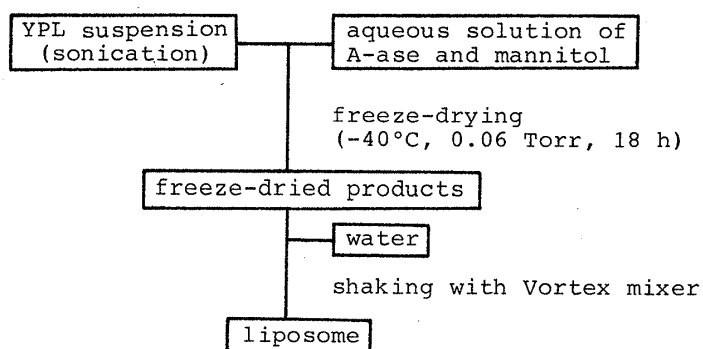


Fig. 1. Preparation of Liposomes by the Freeze-Drying Method

0.45 μm). Two ml of the filtrate was freeze-dried (-40°C , 0.06 Torr, 18 h) in a glass vial and stored at 4°C under nitrogen gas. Then the liposomes were reconstituted by adding 2 ml of distilled water, followed by shaking with a Vortex mixer. For comparison, the liposomes were also prepared by the thin film method:³⁾ A-ase solution in buffered saline (Tris buffer, pH 8.0) was poured into a round bottomed flask coated with YPL film on the inner wall, followed by shaking.

Fig. 2 shows an electron micrograph of negatively stained liposomes prepared by FD method. Liposomes are shown to have a multilamellar structure and their diameters range from 100 to 1000 nm. The smaller vesicles consist of about six concentric bilayers with a large internal aqueous space.

The encapsulating capacity of liposomes prepared by the FD method and the thin film method was compared by determining the latent activity of A-ase using the two methods: a liposomal suspension was approximately diluted with Tris buffer (pH 8.0) and the total A-ase activity (TA) was determined after solubilization with Triton

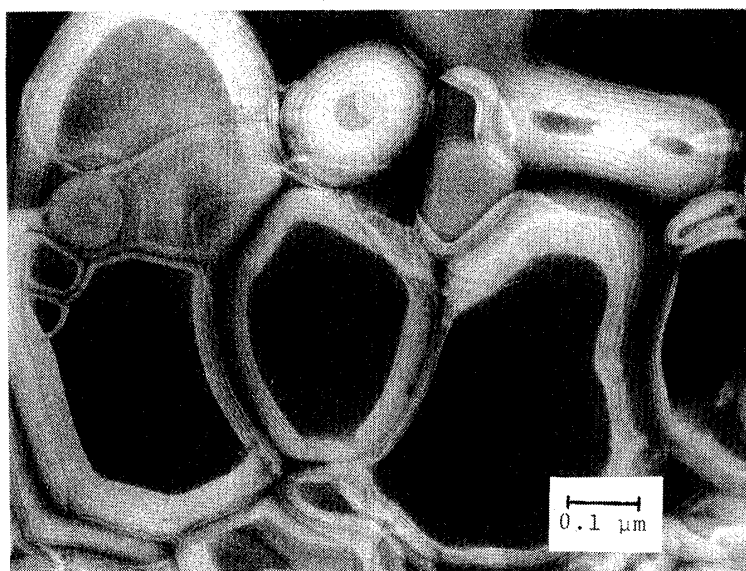


Fig. 2. Electron Micrograph of FD-Liposome Negatively Stained with Sodium Phosphotungstate

X-100. The activity of nonencapsulated free enzyme (FA) was determined without solubilizing the liposomes. The activity was estimated by the modified method of Citri et al.¹⁰⁾ using L-asparagine as a substrate. The calibration curve showed a good proportionality between the amount of liberated ammonia and the A-ase concentration, regardless of the presence of Triton X-100. The encapsulation percentage was calculated by the following equation:

$$\text{Encapsulation (\%)} = 100 \times (\text{TA} - \text{FA}) / \text{TA}$$

Table I summarizes the encapsulation efficiencies of liposomes prepared by the FD and thin film methods. Liposomes prepared by the FD method showed remarkable encapsulating capacity for A-ase, while ordinary liposomes showed low encapsulation efficiency. The encapsulation by liposomes prepared by the FD method increased with the increase of the YPL concentration in the freeze-drying process: approximately 37% and 51% of A-ase was encapsulated at YPL concentrations of 2.5% and 5%, respectively. A-ase was not encapsulated in the liposomes when sonication-dispersed YPL and A-ase solution were mixed without freeze-drying.

In order to find a more convenient procedure for preparing liposomes, they were

Table I. Encapsulation of A-ase in Liposomes Prepared by Freeze-Drying Method

Preparation method	YPL Concentration (%)	Encapsulation (%)
FD Method	2.5	37.4
	5	50.8
Thin film method	5	6.0

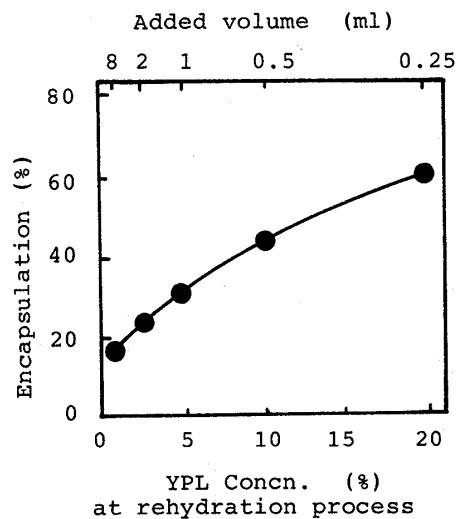


Fig. 3. Effect of Added Volume of Drug Solution on Encapsulation of A-ase in Liposomes, Prepared by Adding A-ase Solution to Prefabricated, A-ase Free, Freeze-Dried Liposomes.

also prepared by adding the drug solution to a prefabricated freeze-dried lipid product. This product was prepared by dissolving mannitol (100 mg) in a sonicated YPL (50 mg) and then freeze-dried. The liposomes were reconstituted by adding different volumes of A-ase solution containing 20 IU. As shown in Fig. 3, A-ase was also encapsulated in liposomes to a relatively high extent and the encapsulation percentage increased with decreasing volumes of A-ase solution.

On the basis of the present observations, it appears that A-ase intruded with water into micro-spaces in the YPL during the rehydration process and was encapsulated in YPL vesicles after the YPL was resuspended with a Vortex mixer. However, further basic study is needed to clarify the mechanism of encapsulation in detail.

The freeze-dry technique has already been used to improve the storage stability of drugs encapsulated in liposomes,¹¹⁾ but significant leakage of hydrophilic drugs from the liposomes occurs during freeze-drying and reconstitution. By contrast, our method probably provides almost the same liposomal product merely by adding the drug solution at the final step of preparation, without any problem of drug stability.

From the viewpoint of pharmaceutical manufacture, the FD method can be considered highly advantageous since it produces liposomes without any residue of organic solvents or detergents and is easily subjected to sterilization and industrial scale-up. The fact that high encapsulating capacity is obtained by the simple manipulation just before use should support the promising applicability of the present method for practical utilization.

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