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ENCAPSULATION OF A WATER SOLUBLE DRUG IN A LIPOSOME PREPARATION: REMOVAL OF FREE DRUG BY WASHING

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ABSTRACT

Multilamellar and unilamellar vesicles were generated by using phosphatidylcholine-phosphatidylglycerol-cholesterol formulation. Liposomes were washed with normal saline to remove non-encapsulated drug. The result of the washing technique was the removal of free (non-encapsulated) drug, in turn improved the percent drug encapsulation in washed liposome preparation. Liposome vesicles were characterized by size, lamellarity, and percent drug encapsulation. Vesicle size was assessed by light scattering, lamellarity by electron microscopy and drug concentration by HPLC techniques.

INTRODUCTION

Liposomes are currently being studied as drug carriers for a variety of drugs which includes recombinant proteins (1-3), and gene transfer and immunodiagnostic applications (4,5). A recent review article pointed out the importance of liposomes in ophthalmic use also (6). The liposomes can protect the drugs from degradation and provide a slow release of drug over a period of time, when they are administered parenterally (7). The therapeutic use of drug-containing liposomes would be greatly aided by the development of methods in which improved drug trapping ability can be achieved. The encapsulation efficiency is defined as the percent of the drug (mg) entrapped in a liposome sample as compared to the total drug in the sample. The free drug remaining

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outside of the liposome is therefore 'wasted' and needs to be removed in order to achieve a better ratio of trapped to non-encapsulated drug in the preparation. Encapsulation efficiency in this report is therefore expressed as percent encapsulation. A detailed characterization of the structure of liposomes with respect to size, morphology, percent encapsulation, and stability on storage is important because it gives information about differences in liposomes structure that affect the behavior of vesicles in *in-vitro* as well as in *in-vivo* conditions.

The simplest way of preparing liposomes is by mechanical dispersion of lipids in water (8). The resulting structures are multilamellar vesicles (MLV) of lipids, which consist concentric bilayers separated by narrow aqueous channels. Further disruption of MLV by sonication (9), extrusion (10), passing through polycarbonate filter (11), use of the Microfluidizer (12), or modifications in hydration techniques can produce unilamellar vesicles (ULV).

A simple washing procedure of the liposomes with normal saline to remove free (unencapsulated) drug from the external media is reported in this paper with encouraging results. In addition, we have described the preparation of "sized" and "unsized" liposomes. We have characterized the liposomes with respect to their encapsulation ability, size distribution, morphology and stability on storage.

MATERIALS AND METHODS

Materials: Lipids used in these studies were egg phosphatidylglycerol, 95% (Avanti Polar Lipids Inc., Birmingham, AL), egg phosphatidylcholine, 95% (Asahi Chemical Industry Company, Ltd., Japan), cholesterol (Croda Inc., Mill Hall, PA) and dl-alpha tocopherol (Sigma Chemicals, St. Louis, MO). Metaproterenol sulfate USP was supplied by Vinchem, Inc., Chatham, NJ. Sodium phosphate monobasic monohydrate, USP and chloroform were supplied by Mallinckrodt Chemical company. All materials were obtained and used as received

Preparation of liposomes: Phosphatidylglycerol, phosphatidylcholine, cholesterol and vitamin E were mixed in the weight ratio of 1:5:2:0.6, in excess amount of chloroform. After evaporation of organic solvent under reduced pressure the thin film of lipids was slowly hydrated with aqueous drug solution (120 mg/mL metaproterenol sulfate). The hydrated liposomes were homogenized with a high shear mixer (Ross & Sons Co.Hauppauge, NY) . The large vesicles so formed were designated as "unsized" liposomes. The liposomes designated as "sized" were further processed by passing through 0.2 μm polycarbonate membrane filters (11). So obtained liposome preparation was concentrated on an ultrafiltration apparatus. The final liposome preparation achieved a paste consistency and was designated as "liposome concentrate".

Washing procedure: The liposome concentrate was diluted 1:3 with normal saline and mixed well to obtain a uniform dispersion of liposomes which was then distributed into ten centrifuge tubes (polycarbonate). Sample volume per

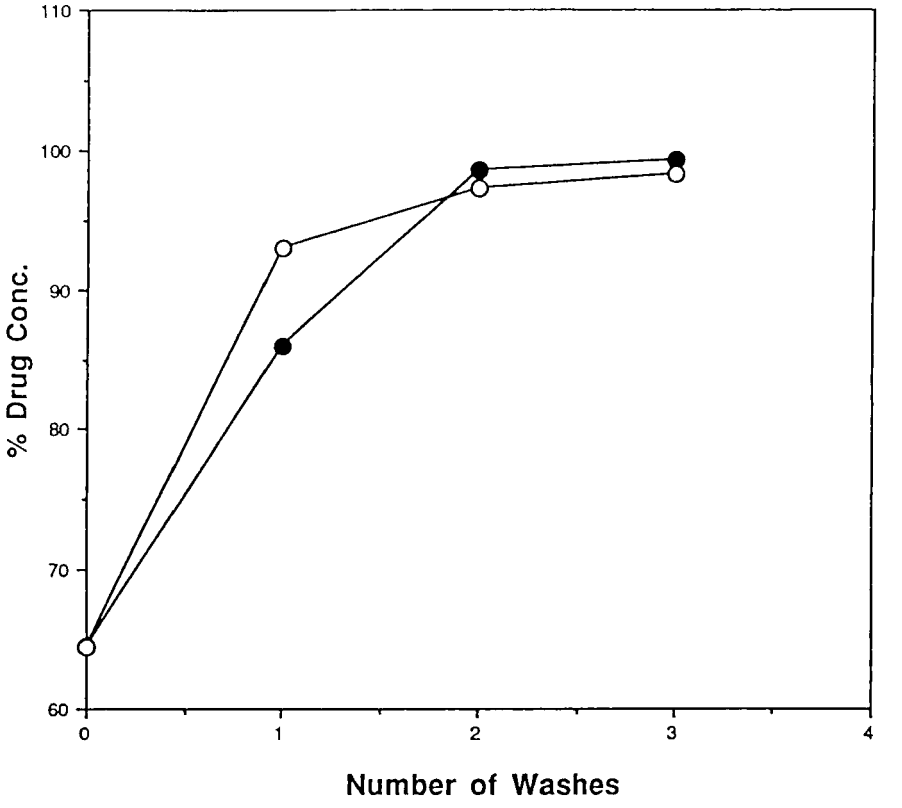

FIGURE 1

Improved drug encapsulation efficiency as a function of washing steps is shown.Two curves in the figure denote two different experiments. Each point in the figure is an average of three determinations and the standard deviation on mean is very small to appear on the points.

tube was limited to 1 mL. The centrifuge tubes were placed in a high speed ultracentrifuge and spun down at 4°C , 100,000 rpm for 30 minutes. This step separated the sample into a firm pellet at the bottom of tubes with a clear supernatant. The supernatant was removed from the pellet by decantation and pipetting off the residual liquid . At this stage the pellet was designated as "one time washed " liposomes . The pellet was then resuspended in 700 µL of fresh normal saline, mixed well, subjected to spinning and separation steps. The resultant pellet is designated as " two time washed " liposomes. The washing procedure was repeated up to three times. The washed pellets were stored at refrigerator (2-8°C) until analysis. Washed liposomes were characterized for size and percent drug encapsulation. Figure 1 shows the effect of normal saline wash on liposome encapsulation efficiency.

Electron microscopy: The freeze-fracture was performed on a Blazers Freeze-Etching System BAF 400D. The micrographs were obtained by using a JOEL JEM-1200 EX Electron Microscope. The samples were cryofixed in the presence of 20-30% glycerol. The fractured surface in electron micrographs was shadowed unidirectionally (45°) with platinum and coated with carbon immediately after fracturing at 163K, 10^{-6} torr.

Vesicle size distribution: Liposome vesicle size was measured on the Nicomp Model 270 Submicron Particle Sizer at 23°C. The magnitude of the turbidity or opalescence depends on the nature, size, and concentration of the particle.

Determination of Encapsulated Drug: An aliquot of liposomes was diluted with normal saline and spun at 10,000 RPM , and 4°C for 30 minutes (Ultracentrifuge, Model TL 100, Beckman Instruments, Inc., Palo Alto, CA). The supernatant and pellet were separated by decantation. The pellet remained in the centrifuge tube was suspended in normal saline and then mixed with an equal volume of 0.5% Triton-X in normal saline. The detergent (Triton-X) lysed the liposomes and yielded clear solution. The resultant clear solution and the supernatant were analyzed by HPLC for drug concentration. Details of HPLC technique are given elsewhere (13). The percentage of drug encapsulated was calculated by the ratio of drug in the pellet to the sum of the drug in the pellet and the drug in the supernatant.

RESULTS AND DISCUSSION

Influence of number of washing steps on % Encapsulation: Washing procedure described in the earlier section was repeated for up to three washing steps. At the end of each washing, a fraction of the total number of centrifuge tubes were set aside for the analysis. The remaining vials were used in the next processing steps. The supernatant and pellet were separated for the analysis. Samples were analyzed for metaproterenol in both supernatant and pellet by a HPLC technique described earlier (13). Precision of the encapsulation efficiency assay on 'twice washed' liposomes was determined to be 96.5% +/- 0.5% (range is 95.66% to 97.41%). Furthermore, in each subsequent experiment, three replicate samples were analyzed for percent encapsulation determination. The data in Figure 1 clearly depicts that the normal saline wash of liposomes removed the external drug from liposome preparation and thereby showed an increase in the percent encapsulation (i.e. ratio of drug concentration inside the liposomes to external media) to greater than 95% by mere two washes. Each point in the figure is an average of three determinations and the standard deviation bars on the points are not evident because the experimental variation was minimal. Both the curves in the figure represent two different experiments.

Influence of lipids concentration on washing : The liposome concentrate was suspended in 1:3 dilution; or 1:4 dilution ; or 1:5 dilution of normal saline. The saline dispersed liposomes were washed once as per the above described process. Samples were analyzed for percent encapsulation, size, and

TABLE 1. Effect of dilution on removal of free drug from external media of a liposome preparation after a single wash. Liposome preparation was diluted with normal saline.

Dilution Liposome: Normal Saline	Drug Encapsulation (%)+/-S.D.	Drug in supernatant (mg/ml)
1:0 (control)	64.40 +/- 0.1	------
1:3	83.80 +/- 0.2	10.96
1:4	85.86 +/- 0.07	10.08
1:5	91. 30 +/- 0.2	5.44

TABLE 2. Characteristics of "unsized" and "sized" liposomes after washing process.Standard deviation (S.D.) on the mean for three determination is given.

Parameter	Unsized Liposomes	Sized Liposomes
Drug concentration +/- S.D	39.8 +/- 0.9	56.1 +/- 1.0
Percent Encapsulation +/- S.D.	96.5 +/- 0.5	94.8 +/- 0.2
Vesicle size (μm) +/- S.D.	2.410 +/-1.094	0.156 +/- 0.093

drug concentration. As expected, the results in Table 1 indicate that when the liposome preparation was diluted with more normal saline (i.e. 1:5) the free drug in external media was removed more efficiently as compared to the other dilutions tried in this study.

Characterization of "Unsized" and "Sized" liposomes after Washing: Liposomal preparations can be adequately characterized by lamellarity, size and trapped volume. In this study trapped volume was indirectly measured as percent encapsulation. Size of liposomes was assessed by freeze-fracture electron microscopy and by quasi elastic light scattering techniques. The vesicle size determined in our experiments, ranged from 1-32 μm for "unsized" and 0.15 to 0.34 μm for "sized" liposomes. Both sized and unsized liposomes were twice washed to remove the free drug. The expected end result of the washing procedure was increased percent drug encapsulation in the preparation. Table 2 shows the quantity of drug in liposomes, percent drug encapsulated and the average vesicle diameter for both unsized and sized liposomes. As expected unsized vesicles encapsulated less drug as compared to sized vesicles. In both

FIGURE 2

Freeze-fracture micrographs of liposomes before and after sizing is shown. (A) Multilamellar vesicle showing 'onion skin configuration. (B) Sized vesicles are small and larger space to hold drug solution. The horizontal bar in the right hand side corner of micrograph represents 500 nm in length.

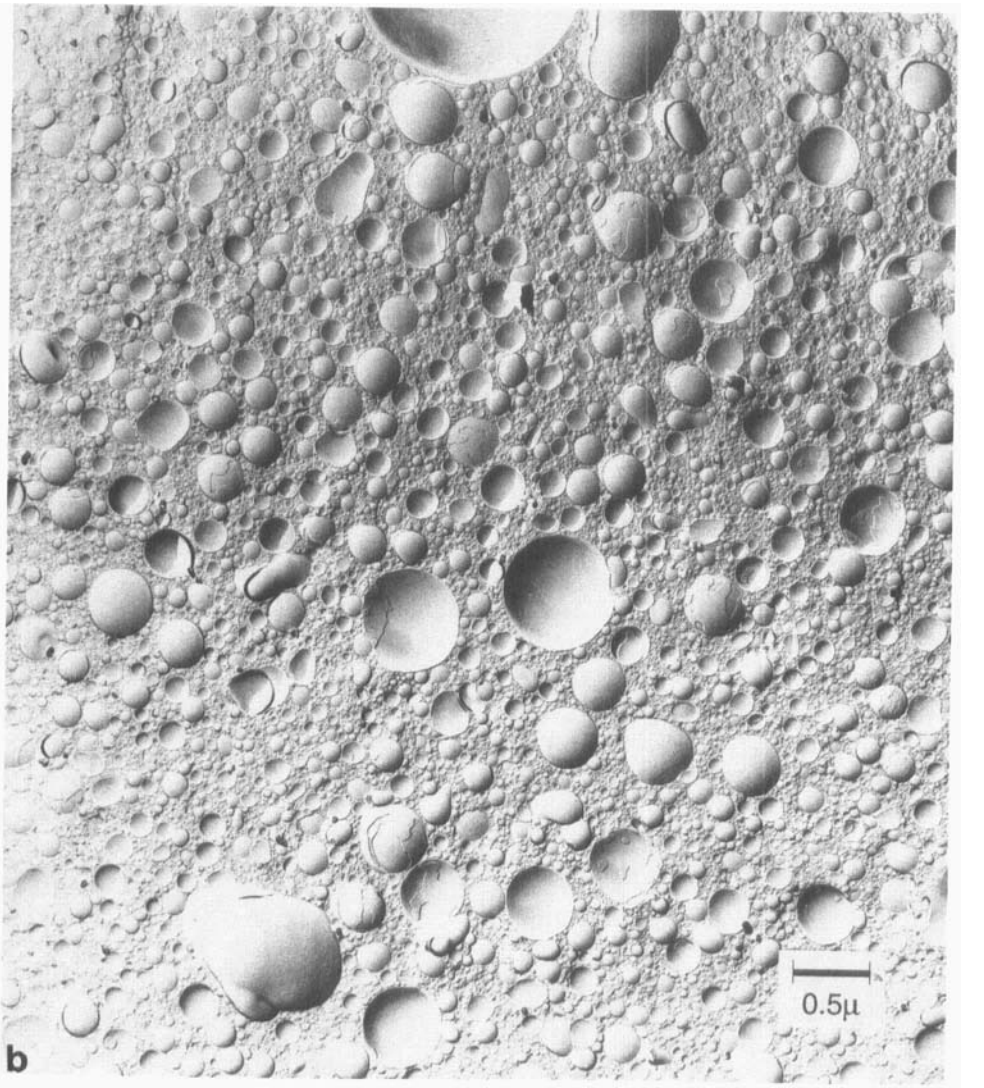

FIGURE 2. Continued

TABLE 3. Stability of twice washed liposomes stored at 2-8°C in USP Type I glass containers. Standard deviation (S.D.) on the mean for three determinations is given.

Time (weeks)	Particle size (μm) +/- S.D.	Encapsulation (%) +/- S.D.	Total Drug (mg/g lipid)+/-S.D.
0	0.201+/- 0.002	96.7 +/- 0.2	66.0 +/- 1.0
2	0.200 +/- 0.004	96.5 +/- 0.7	64.4 +/- 0.1
4	0.199 +/- 0.001	95.2 +/- 0.8	63.0 +/- 2.0
12	0.200 +/- 0.010	96.3 +/- 0.7	61.0 +/- 3.0

cases, percentage encapsulation was as high as 95. The morphology of liposomes was examined by electron microscopy technique. As shown in the electron micrograph (Fig. 2A) the unsized liposomes exhibited a tightly packed "onion skin" configuration of bilayers with relatively less space available for drug solution to remain in the vesicle. The interlamellar space between bilayers carry limited amount of aqueous drug solution as compared to unilamellar vesicles. The larger vesicles are as big as 10 μm in size with several shells of bilayers. On the other hand the sized vesicles showed (Fig 2B) relatively larger space inside the vesicle for drug solution to reside. The electron micrographs showed that the small vesicles were ranging from about 0.025-2.5 μm in size. The small vesicles contain probably one or two shells of bilayers. Mayer et. al. (14) correlated the vesicle size determined by the quasi elastic light scattering method to the vesicle lamellarity determined by the ^{31}P NMR technique. According to this study the diameter of 0.18 +/- 0.15 μm indicate unilamellar vesicles or bilamellar vesicles (BLV). Based upon this information, the sized liposomes (0.246+/-0.093 μm) can be characterized as ULV or BLV while the unsized (2.146 +/- 1.094 μm) liposomes are MLV in character.

Stability of Washed Liposomes: A batch of sized and washed liposomes (pellet) were stored at 2°-8°C. At the end of 4, 8, and 12 weeks, the stored samples were analyzed for percent encapsulation, total drug, and vesicle size. Results are shown in the Tables 3. Furthermore, drug release profile of 12 week stability samples were compared with zero time samples. Drug release profiles determined over 8 hour period for zero-time and 12 week stability points were very similar (data not shown). The data presented in the Table 3 and drug release data suggest that the washed liposomes maintained their physical integrity and chemical potency throughout the test period.

CONCLUSION

A simple washing procedure efficiently removed the "free" drug from a liposome preparation made of phosphatidylcholine-phospatidylglycerol-

Cholesterol. Liposomes washed with normal saline for three times removed more than 95% of "free" drug which is evidenced by an encapsulation of greater than 97%. Liposomes diluted with more volume of normal saline removed the free drug more efficiently than the lesser volumes. This washing procedure was performed in a high speed, laboratory model centrifuge. The utility of this technique in large scale production merits further study. Washed liposomes showed a good stability at 2-8°C through out the test period.

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