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The use of a high intensity ultrasonic processor equipped with a flow cell in the production of reverse-phase liposomes

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Abstract

A high intensity ultrasonic processor equipped with a flow cell (Vibra Cell®) was used in the production of reverse-phase liposomes. The size distribution of the lipsosomes was determined with negative stain electron micrographs and the mean diameter of the liposomes was found to be 234 nm. The internal volume was 18.26 l of aqueous phase per mol of lipid and the encapsulation efficiency was 33.57%. The use of the proposed flow cell in connection with the reverse-phase evaporation method ensures uniform production of liposomes with good reproducibility in internal volume and encapsulation efficiency.

Keywords: Probe sonicator; Flow cell; Reverse-phase liposome; Size distribution; Internal volume; Encapsulation efficiency

1. Introduction

Interest in the use of liposomes as drug carriers in the field of dermatological treatment has been increasing during recent years. Accumulating evidence shows that liposomes have the potential to improve the therapeutic efficacy of topically applied drugs. In general, liposomal encapsulation appears to give a higher drug concentration at the intended site of action and thereby enhance the local therapeutic effect but at the same time minimize the unwanted systemic side effects (Mezei, 1985). The way the liposomes deliver the drug into the skin is not very clear. Some predict that the liposomes partly or totally rupture at the skin surface (Junginger et al., 1991; Plessis et al., 1994) while others postulate that liposomes are able to penetrate the skin intact (Foldvari et al., 1990). When studying the penetration of liposomes into the skin, Foldvari et al. (1990) determined that there is a size restriction for the penetration of intact liposomes into the skin. Their work revealed that the diameter of the liposomes should not exceed 500 nm to ensure effective dermal delivery of the liposomal drug (Foldvari et al., 1990). Recently, Plessis et al. (1994) investigated the influence of liposomal size on drug deposition into the skin. Their work demonstrated that drug delivery is not only influ-

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enced by the diameter of the liposomes but there also seems to be an 'optimum' vesicle size for maximal drug delivery, for example, liposomes with a mean diameter of 300 nm delivered a higher drug concentration into the deeper layers of the stratum corneum than smaller liposomes with a mean diameter of 60 nm (Plessis et al., 1994).

When choosing a suitable method in the production of liposomes to carry water-soluble substances, reverse-phase evaporation is in many cases the preferred way. Reverse-phase liposomes have much higher encapsulation efficiency for water-soluble drugs than other types of vesicles like multilamellar liposomes (Szoka and Papahadjopoulos, 1978, 1981).

The purpose of the present work was to investigate the use of a probe-type sonicator, equipped with a flow cell, for the formation of the o/w emulsion in the reverse-phase evaporation method. The object was to ascertain whether these modifications could give liposomes with a diameter smaller than 500 nm and high reproducibility in both internal volume and encapsulation efficiency.

2. Materials and methods

2.1. Materials

Liposomes were made from soy lecithin as a phospholipid (Phospholipon 90H, Natterman phospholipide GmbH, Cologne, Germany) and cholesterol (approx. 95% anhydrous, Sigma Chemical Co., St. Louis, MO). Sodium salicylate was obtained from Apodan, Copenhagen, Denmark.

2.2. Preparation of liposomes

Reverse-phase evaporation vesicles were prepared by the reverse-evaporation method (Szoka and Papahadjopoulos, 1978, 1981). Briefly, phospholipid (PL 90H) and cholesterol in a 2:1 molar ratio were dissolved in an organic solvent (dichloromethane/methanol, 1:1). The aqueous phase, containing sodium salicylate as a model substance for water-soluble compounds, was then added and the solution gently stirred until it became homogeneous. The solution was then run at a rate of about 2 ml/min through a flow cell and sonicated at maximum output with 50% duty cycle (see Fig. 1). The sonication step was repeated twice to ensure optimal size distribution of the liposomes. The organic phase was then evaporated from the solution in a rotavapor connected to a vacuum pump (Millipore®), first at low pressure, about 300 mmHg, to inhibit the emulsion from refluxing into the rotavapor. The pressure was then gradually increased until full vacuum was obtained. The water bath was kept at 48°C. The final composition of the three liposomal batches is listed in Table 1.

2.3. Characterization of liposomes

2.3.1. Size distribution

The liposomal size distribution was characterized with negative-stain electron micrographs (Philips EM 300 TM electron microscope). Briefly, 1 ml of the liposomal suspension was diluted with iso-osmotic sodium chloride solution until the final phospholipid concentration in the sample was about 0,25%. Standard 200 mesh copper-coated electron microscope grids (Athene grids, Ted Pella Inc., Redding, CA) with formvar film

Table 1
Final composition of the three liposomal batches

Batch [a]	PL90H		Cholesterol		Sodium salicylate % (v/w) [b]
	mg	μmol/ml	mg	μmol/ml	
1	84.6	9.56	22.1	5.06	0.367
2	84.4	9.21	22.0	4.90	0.355
3	84.7	9.57	22.1	5.10	0.367

[a] The final volume of batches 1 and 3 was 11.3 ml and 11.7 ml for batch 2.
[b] Each batch contained about 0.160% sodium chloride.

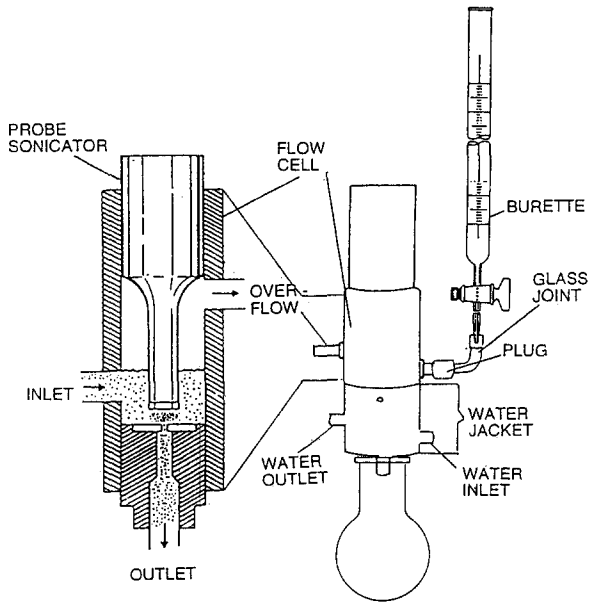

Fig. 1. Probe sonicator connected with a flow cell (schematic sketch).

were used. One drop of diluted liposomal suspension was placed over the formvar coated side of the grid for 60 s. The drop was then, according to the drop method described by Bulgeski et al. (1990), drawn off the grid with a piece of filter paper and one drop of 2% uranyl acetate solution placed onto the grid. After exactly 60 s the drop was drawn off and the grid was allowed to dry thoroughly (Bulgeski et al., 1990).

In order to determine the mean diameter of the liposomes more than 400 particles were counted to ensure accurate statistical evaluation of the liposomal size distribution (Martin et al., 1989).

2.3.2. Internal volume

In determining the internal volume, a 2 ml sample of the liposomal suspension was centrifuged at $3020 \times g$ for 30 min (ICE centra-3 centrifuge) at room temperature. The supernatant was then carefully removed with a 20 gauge needle to prevent any material from being discharged along with the supernatant. A volume of iso-osmotic sodium chloride solution equal to that of the previously removed supernatant was then replaced in the liposomal pellet, and the solution gently shaken and then centrifuged as before. This washing procedure was repeated three times to ensure that unencapsulated sodium salicylate was no longer present in the void volume between the liposomes. The pellet was then dissolved in anhydrous ethanol and the concentration of sodium salicylate (X_1) was determined at 295.5 nm with a Perkin Elmer 550 SE UV/Vis spectrophotometer. The molecular concentration of sodium salicylate in the supernatant ($C_{\text{supern.}}$) was also determined spectrophotometrically and the internal volume (V_1) was evaluated according to the formula:

$$V_1 = V_T - \frac{(X_T - X_1)}{C_{\text{supern.}}}$$

where V_T is the total volume of the liposomal suspension and X_T denotes the total mol content in the liposomal suspension.

2.3.3. Encapsulation efficiency

Drug encapsulation was determined according to Foldvari et al. (1993), i.e., the encapsulation efficiency was determined from the molecular concentration of sodium salicylate in the supernatant and the liposomal pellet, as previously described.

$$\%\ \text{encapsulation}: \frac{C_{\text{pellet}}}{C_{\text{pellet}} + C_{\text{supern.}}}$$

3. Results

The results for the determination of the encapsulation efficiency and internal volume are shown in Table 2. The average internal volume of

Table 2
Internal volume and encapsulation efficiency of the liposomes

Batch	Internal volume		Encapsulation efficiency (%)
	l/μmol	ml	
1	18.45 ± 0.33	3.08 ± 0.05	36.18 ± 0.32
2	17.34 ± 0.09	2.86 ± 0.01	33.72 ± 0.11
3	19.10 ± 0.12	3.16 ± 0.02	30.82 ± 0.16
Average	18.26	3.03	33.57

Fig. 2. Negative-stain electron micrograph of REV liposomes. Bar indicates 500 nm.

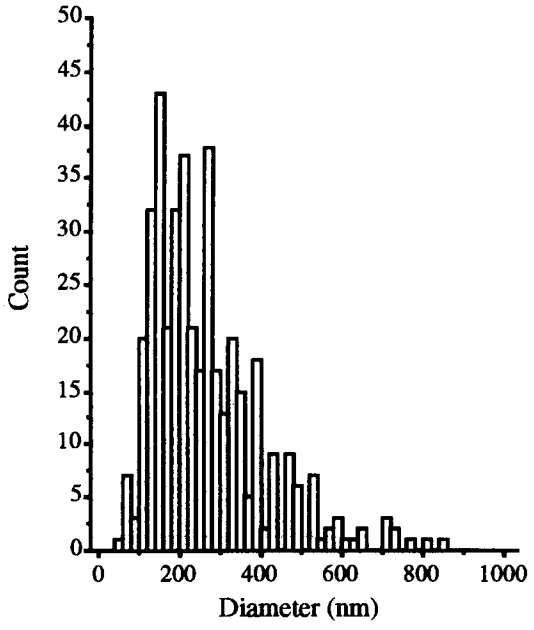

Fig. 3. Size distribution of the liposomes.

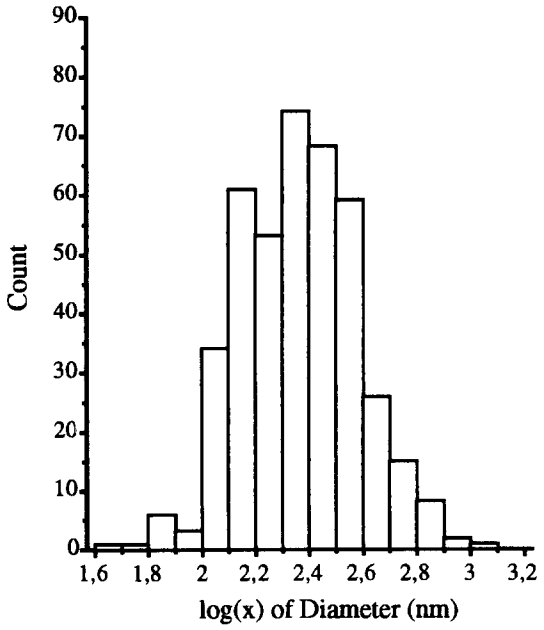

Fig. 4. Log transformation of the liposomal diameter.

three liposomal batches was found to be 18.26 l of aqueous phase per mol lipid, and the average encapsulation efficiency to be 33.57%. From the electron micrographs it was established that about 70% of the liposomes had a diameter smaller than 300 nm (see Fig. 2 and 3). The lipsomal size distribution was clearly skewed to the right and therefore the data were transformed to a log-normal distribution (see Fig. 4). From the log-normal size distribution the mean diameter of the liposomes was determined as 234 nm.

The flow cell offers the opportunity of circumventing many problems associated which the use of probe sonicators, like heat production and aerosol generation, but the difficulties of preventing the possible shedding of metal particles from the probe tip remain to be resolved.

4. Conclusion

It can be concluded from this work that the production of REV liposomes by using a high intensity ultrasonic processor equipped with flow cell is a feasible option to manufacture REV liposomes with a narrow size distribution well within the 500 nm diameter limit. Good reproducibility was found in the internal volume between batches and the reproducibility of the encapsulation efficiency between batches was very acceptable.

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