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Preparation of liposomes using a Mini-Lab 8.30 H high-pressure homogenizer

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Summary

The range of liposome preparation methods has recently been extended by a number of techniques which are based on the use of homogenizers. However, only a few suitable types of apparatus have been proposed so far. This report describes work with a continously working Mini-Lab 8.30 H high-pressure homogenizer of APV Rannie. Homogenization of dispersions of preformed vesicles which were multilamellar and heterogeneous in size resulted in uniform, small unilamellar vesicles (SUVs). The previously described one-step method (Brandl et al., *Drug Dev. Ind. Pharm.*, 16 (1990) 2167–2191) for liposome preparation could also be carried out using the Mini-Lab homogenizer; the resulting vesicles were again very small and homogeneous after one cycle only. Higher homogenization pressures and repeated recirculation led to further reduction in vesicle diameter and heterogeneity. Finally, after about 15 cycles size distributions with peaks of about 30 nm and upper limits below 100 nm could be achieved. With higher lipid concentrations (maximum of 100 mg/ml) size reduction by homogenization was less effective. In the case of cholesterol-containing vesicles, maximum size reduction during repeated homogenization was observed after five to ten cycles. Further recirculation caused vesicle re-growth. The encapsulation efficiency of one-step vesicles for carboxyfluorescein at lipid concentrations (100 mg/ml) was nearly 12%, the entrapped volume being about 0.90 l/mol lipid. Repeated homogenization (up to 30 cycles) reduced these values by about 50%. The erosion of the most stressed part of the apparatus (i.e., the valve) was low, as monitored microscopically. During homogenization a slight leakage from the product compartment into the cooling water compartment was observed. Leakage of cooling water into the product compartment could be seen only during standby periods.

Introduction

Phospholipid vesicles consisting of an aqueous compartment surrounded by one or more bilayer membranes are under investigation as cell membrane models, as carriers for drugs and bioactive molecules and as immunological adjuvants in vaccines (Gregoriadis, 1988a). Whilst the characteristics of the bilayer itself are mainly determined by the nature of the lipid(s) used, the type of vesicles (uni- or multilamellar), the vesicle sizes and the extent of entrapment of water-soluble molecules are primarily influenced by the chosen method of preparation. A range of methods have been described so far (e.g., Bangham et al., 1965; Batzri and Korn, 1973; Szoka and Papahadjopoulos, 1978; Kirby and Gregoriadis, 1984; Weder

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and Zumbuehl, 1984; Shew and Deamer, 1985). Since industrial scale production of liposomes has become reality in some cases, methods which use mixing devices such as high-pressure homogenizers and microfluidizers are increasingly attracting interest (Mayhew et al., 1984; Talsma et al., 1989; Brandl et al., 1990, 1993; Gregoriadis et al., 1990; Mentrup and Stricker, 1990). The main advantages of homogenized liposomes are their single bilayer membrane and small and homogeneous vesicle size. The processes work mostly under mild conditions, are cost-effective and may be scaled up easily. We have previously used a double-jet homogenizer (Microfluidizer M 110, Microfluidics Corp.) for the reduction of lamellarity and sizes of preformed dehydration-rehydration vesicles, which are capable of entrapping solutes very effectively (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1990). For the development of a method which produces SUVs from powdered lipid(s) and aqueous drug solution in just one step (Brandl et al., 1990), we used a discontinously working laboratory-scale Micron Lab 40 ultra-high-pressure homogenizer (capacity 40 ml/batch) of APV Gaulin, Lübeck, Germany. Experiments designed to transform the one-step method to the production scale were performed on a continously working Gaulin Lab 60 with a capacity of 60 l/h (Brandl et al., 1993). The intention of the present study was to evaluate the suitability of an apparatus of the APV-Rannie series, the continuously working Mini-Lab 8.30 H laboratory homogenizer (APV Rannie, Albertslund, Denmark) for liposome formation according to the regulations of 'good manufacturing practices', which are in operation in pharmaceutic production. During the tests, not only size reduction of preformed vesicles, but also liposome formation via the one-step method were carried out. The encapsulation efficiency was determined by assaying carboxyfluorescein associated with liposomes.

Materials and Methods

A highly purified phosphatidylcholine fraction of soy bean lecithin (SPC) (Phospholipon 90) was obtained from Nattermann Phospholipid GmbH, Cologne, Germany. Cholesterol (Ch), Triton X-100 and methylene blue were purchased from BDH, Poole, U.K. Carboxyfluorescein (CF) was from Eastman Kodak and purified by molecular sieve chromatography on a Sephadex LH 20 (Pharmacia, Uppsala, Sweden) column (Kirby et al., 1980).

The homogenizer used (Mini Lab 8.30 H) consists of a peristaltic pump which feeds the material into a water-cooled two-piston high-pressure pump. The hand-wheel-operated valve comprises a small ceramic ball and a steel seat. Maximum pressure in our experiments was 100 MPa, which is the recommended upper limit. We first exchanged the plastic funnel supplied as inlet reservoir with a separation funnel made of glass and the tubing for recirculation with a silicone tube of 8 mm inner diameter. With these changes the minimum operating volume could be reduced from 120 ml to less than 100 ml. All experiments were performed with 100 ml batches in a continuous recirculation mode.

For homogenization, preformed vesicle dispersions were generated by the method of Bangham (1965). Alternatively, mixtures of water with lipid(s) were homogenized directly (one-step method). Liposome sizes were analysed using a Malvern Autosizer 2.2. On the basis of findings from previous experiments (Brandl et al., 1993) that size distributions of homogenized vesicles are mostly non-Gaussian and cannot therefore be characterized properly by arithmetic mean and standard deviation, we decided to use instead the vesicle diameter (D) which represents the peak of the equivalent normal weight distribution curve and the polydispersity index (PI). Determination of encapsulation efficiencies was carried out using the water-soluble marker CF at a concentration of 6.64 μmol/l. Following separation of the non-encapsulated CF by size exclusion chromatography on a Sephadex G-50 (Pharmacia) column and Triton X-100-mediated lysis of the vesicles, their CF content was determined fluorimetrically (Perkin Elmer 204 A; excitation at 490 nm, emission at 520 nm) against standard curves generated from mixtures of CF, empty vesicles and Triton X-100.

TABLE 1

Vesicle diameters and polydispersity indices of vesicles prepared according to the one-step method

Lipid(s) used	Lipid conc. (mg/ml)	Press. (MPa)	Number of cycles									
			1		2		5		10		15	
			D (nm)	PI	D (nm)	PI	D (nm)	PI	D (nm)	PI	D (nm)	PI
SPC	10	40	63	0.46	63	0.41	49	0.42	36	0.43	31	0.36
SPC	50	40	71	0.44	54	0.45	41	0.53	31	0.51	26	0.46
SPC	100	40	111	0.36	78	0.39	64	0.44	52	0.43	35	0.46
SPC	100	70	77	0.42	65	0.42	45	0.52	34	0.48	26	0.47
SPC + Ch	100	70	73	0.42	54	0.45	40	0.42	45	0.33	57	0.24

Lipid conc., total concentration of lipid(s); Press., homogenization pressure; *D*, vesicle diameter; PI, polydispersity index.

Results and Discussion

Liposome preparation

We first homogenized preformed hand-shaken liposomes under moderate pressure (40 MPa). A single cycle transformed the raw vesicle dispersion into a homogeneous opalescent to slightly turbid dispersion. We also employed powder phospholipid (SPC) and water (10–100 mg lipid per ml). After recirculating without pressure (open valve) for a few minutes, a pressure of 40 MPa was applied for one cycle. As with the first approach, homogeneous opalescent to slightly turbid dispersions were obtained. Lipid-water mixtures with lipid content of up to 100 mg/ml could be processed. Only when the starting mixture contained coarse lipid particles of more than 2 mm in diameter was homogenization ineffective. This was due to blockage of one of the back-pressure valves which necessitated the dismantling and cleaning of the apparatus.

Following the one-step liposome preparation samples were drawn at intervals during recirculation and analysed for vesicle sizes (Table 1).

After the first cycle, 10 mg/ml of the SPC vesicles were quite small and homogeneous in size with distribution peak diameters of about 60 nm and upper limit diameters of about 200 nm. Recirculation led to further reduction in size with distribution peaks as low as 30 nm and upper limits below 100 nm after 15 cycles. Similar results were obtained with 50 mg/ml dispersions. However, 100 mg/ml dispersions, which are more viscous, contained larger vesicles after the first and subsequent few cycles. After 15 cycles vesicle sizes were very similar to those in the more dilute dispersions. When the experiment with SPC was repeated by applying a homogenization pressure of 70 MPa instead of 40 MPa, the vesicles obtained were generally smaller (Table 1). After extensive recirculation, however, such differences in size decreased.

TABLE 2

Encapsulation efficiency and entrapped aqueous volume of vesicles prepared according to the one-step method

Press. (MPa)	Number of cycles											
	1		3		5		10		20		30	
	Eff. (%)	Volume (1/mol)	Eff. (%)	Volume (1/mol)	Eff. (%)	Volume (1/mol)	Eff. (%)	Volume (1/mol)	Eff. (%)	Volume (1/mol)	Eff. (%)	Volume (1/mol)
40	11.8	0.91	n.d.	n.d.	10.4	0.80	8.0	0.62	7.6	0.58	7.0	0.54
70	11.6	0.89	11.3	0.87	9.0	0.69	7.5	0.58	6.1	0.47	n.d.	n.d.

Press., homogenization pressure; Eff., encapsulation efficiency; Volume, entrapped aqueous volume.

Using vesicles made of equimolar Ch and SPC, sizes again first decreased during recirculation to a minimum of 40 nm after about five cycles. Interestingly, vesicle size tended to increase when 10 or more cycles were used. Similar findings have been reported before with the Gaulin Micron Lab 40 homogenizer (Brandl et al., 1990) and the Gaulin Lab 60 homogenizer (Brandl et al., 1993), and experiments are underway to study this phenomenon.

Encapsulation efficiency

Values of CF and volume entrapment in vesicles after homogenization of SPC (100 mg/ml) with CF solution are listed in Table 2. Entrapment values for CF were nearly 12% after one cycle at 40 or 70 MPa. The corresponding aqueous volumes entrapped in vesicles after one cycle were 0.89 and 0.90 l/mol lipid. During recirculation, however, entrapment values were reduced to 7% and 0.54 l/mol at 30 cycles.

Table 3 compares the entrapment efficiency (%) and entrapped aqueous volume (l/mol) values of the present one-step method of vesicle preparation with literature data for vesicles obtained by other methods. Although studies shown are based on a variety of markers, they can be considered as comparable because all markers are water soluble and of low and similar molecular weight. Since higher lipid concentrations lead to higher encapsulation efficiencies, the amounts of phospholipid used in the different preparations shown are also given. It is apparent that the method of ethanol injection and that of microfluidization result in entrapped aqueous volumes of the same magnitude as that observed with the one-step method using the Mini Lab 8.30 H. Further, the encapsulation efficiencies (6.3 and 11.6%) achieved with the microfluidization and one-step methods are comparable if the concentrations of the lipids used are taken into account. In contrast, the dehydration/rehydration (DRV) and reverse-phase evaporation (REV) methods entrap water-soluble molecules much more efficiently (54 and 55% for the examples of solutes given) and with the REV method the aqueous space (13.7 μl/mg) is also much greater than that of the one-step method (1.20 μl/mg). Such differences in entrapment values are due to the small size of vesicles obtained with the ethanol injection, detergent dialysis and homogenization techniques as opposed to the large unilamellar/oligolamellar type of vesicles obtained with REV/DRV methods.

General performance and problems of the method

The homogenization pressure applied during the procedure can be directly followed on a di-

TABLE 3

Entrapment values and vesicle characteristics obtained by various methods of liposome preparation

Method (reference)	Lipid(s) used	Conc. (μmol/ml)	Marker	Marker entrap. (%)	Volume entrapped		Mean size (nm)	Lamellarity
					(l/mol)	(μl/mg)		
One-step method [a]	SPC	135	CF	11.6	0.89	1.20	$D = 77$	unilamellar
Ethanol injection [b]	EPC	< 100	Gluc	n.d.	0.52	–	26.5	unilamellar
Microfluidization [c,h]	PC/PG/Ch	60	AraC	6.3	1.03	–	< 200	unilamellar
		180	AraC	17.4	0.97	–		
Detergent dialysis [d]	EPC	65	Inulin	12.4	n.d.	–	65	unilamellar
REV [e]	PC/PG/Ch	66	AraC	55.0	–	13.7	460	uni-/oligol.
DRV [f]	EPC	16.5 [g]	CF	54.0	n.d.	–	300	uni-/oligol.

[a] Brandl et al. (1990); [b] Batzri and Korn (1973); [c] Mayhew et al. (1984); [d] Weder and Zumbuehl (1984); [e] Szoka and Papahadjopoulos (1978); [f] Kirby and Gregoriadis (1984). [g] Although the concentration of lipid is 10-fold higher at the stage of rehydration and vesicle formation, the actual amount of lipid used is still relatively low. [h] After 2 min microfluidization at about 70 MPa. REV, reverse-phase evaporation vesicles; DRV, dehydration/rehydration vesicles; EPC, egg phosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; Ch, cholesterol; CF, carboxyfluorescein; AraC, [^{3}H]cytosine arabinoside; Gluc, radiolabelled glucose.

aphragm pressure gauge. This is conducive to good reproducibility of preparations. However, when high pressures are applied during operation, it is often difficult to avoid leakage from the sealings and packings. In order to detect leakage from the product compartment into the cooling water compartment, a solution of methylene blue was processed at 18 MPa for 15 min (which corresponds to about 25 cycles of 100 ml). Total leakage of 0.68 ml was detected. After changing all rubber sealings and packings the leakage was still about 0.62 ml. Conversely, considerable leakage of cooling water into our product was observed as an increase in volume when the tap for the cooling water was open during standby periods, i.e., when the homogenizing valve was open and the pressure was down to values of zero.

Because of the extreme forces present in high-pressure homogenizers, abrasion can be a serious problem with particulate material contaminating the product. However, this could not be detected by centrifuging the product. Only by monitoring the most stressed parts of the valve, the steel seat and the ceramic ball, could faint ring-shaped traces of erosion be seen (Fig. 1). When routine cleaning was carried out according to the instructions of the manufacturer by recirculating water and detergents, traces of lipid were occasionally found, especially in the valves. For operations according to GMP dismantling, cleaning, drying and reassembling the valve housing after every use is recommended.

Fig. 1. Microscopic detail (magnification 50-fold) of the surface of the ceramic ball which forms the mobile part of the valve.

In conclusion, the general performance of the Mini Lab 8.30 H high-pressure homogenizer in terms of homogenization effectiveness, pressure constancy, accessibility for inspection and cleaning is good. The Mini Lab 8.30 H is able to process mixtures of water and lipid(s) (up to a concentration of at least 100 mg/ml) without any pretreatment. The lipid particles, if smaller than about 2 mm, are effectively broken up and hydrated subsequently to form small vesicles.

The homogenization efficiency is better with higher pressures, as can be seen from the smaller vesicle diameters and narrower size distributions obtained. A similar effect is achieved by recycling the product up to 10 times. However, 10 or more cycles cause, at least in the case of cholesterol-containing vesicles, an increase in size.

The amount of entrapped CF is of the same magnitude when compared to other preparation methods for SUVs. However, compared to techniques which lead to larger vesicles, for instance, those of the REV or DRV methods, entrapment is low. On the other hand, small and homogeneous vesicles are known to have longer circulation half-lives when injected intravenously, especially when phospholipids with high gel-liquid crystalline transition temperatures are used (Gregoriadis, 1988b). As expected, high homogenization pressures and repeated processing, which reduce vesicle size, also decrease the entrapped volume and encapsulation efficiencies.

The Mini Lab 8.30 H is very suitable for the rapid production of quite concentrated dispersions of very small and uniform liposomes under relatively mild conditions, regardless of the lipid(s) used. Further, as all parts of the apparatus that come into contact with the product can be dismantled, thoroughly cleaned and, if necessary, sterilized, standards of sterility as required in GMP seem to be attainable.

The only serious drawback is the leakage of product into the cooling water and leakage of cooling (lubricating) water into the product. The latter can largely be avoided if the cooling system is not used during the standby periods.

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