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THE SIZE REDUCTION OF LIPOSOMES WITH A HIGH PRESSURE HOMOGENIZER (MICROFLUIDIZER™). CHARACTERIZATION OF PREPARED DISPERSIONS AND COMPARISON WITH CONVENTIONAL METHODS.

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ABSTRACT

A high pressure homogenizer (the Microfluidizer™) was tested for its ability to produce liposomal dispersions with a narrow size distribution. For dispersions with 40 μmol phospholipid/ml, sonication and high pressure homogenization gave similar average particle diameters. With the Microfluidizer™ it was possible to produce reproducible dispersions with a mean particle size between 0.05 and 0.25 μm and a narrow size distribution containing up to 200 μmol phospholipid/ml. However, after processing 50 ml batches significant lipid loss was observed and small amounts of metal particles were found in the dispersions.

INTRODUCTION

Liposomes are currently under investigation as drug delivery systems for the controlled release of drugs or for drug targeting. A proper

characterization of the vesicles is desired, because differences in liposome charge, bilayer rigidity, size and inner structure result in differences in pharmacokinetic behavior in vivo after injection[e.g. 1,2,3]. Recently, we published a study in which we discussed appropriate physico-chemical techniques for liposome characterization [4].

Numerous techniques have been developed for the lab-scale production of well defined liposomes[e.g. 2,5]. However most of them are not suitable for the large scale production of liposomal dispersions. There is a need for reliable techniques to produce liposome dispersions with narrow particle size distributions on a (semi)-industrial scale reproducibly.

We tested a high pressure homogenizer (the Microfluidizer™), an apparatus claimed to produce relatively large batches of egg phosphatidylcholine containing vesicles with relatively narrow size distributions[6,7,8]. In this study the composition of the bilayer -and thereby the rigidity and surface potential- and the initial concentration of the lipids in the dispersion were varied. Size, size distribution of the produced vesicles and zeta-potential were measured.

The results were compared with two lab-scale liposome production methods to obtain narrow particle size distributions: liposomes prepared with the "film"-method and thereafter extruded or ultrasonicated.

MATERIALS AND METHODS

Materials

Phospholipon 100 "low Lyso"(PL100), composition over 90% soybean phosphatidylcholine, about 5% free fatty acids and/or lyso-lipids and 1.2% water and Phospholipon 100H (PL100H), composition over 94% hydrogenated soybean phosphatidylcholine, less than 0.5% lyso-phospholipid and 1% water were supplied by Nattermann (Cologne, F.R.G). Cholesterylhemisuccinate Tris salt (CHEMS) was obtained from Sigma Chemicals (St.Louis, MO). All other chemicals used were of analytical grade.

Preparation of the Liposomes

Three methods for narrowing down the particle size distribution were tested. The raw dispersions were prepared according to the "film method" and subsequently extruded, sonicated or treated with the Microfluidizer™. Extrusion was performed through 0.6 and 0.2 µm Unipore™ filters (Uni-pore, Bio-Rad, Richmond, CA). Sonication was performed with a Bransonic B12, probe type sonicator (Branson Inst. Comp., Stamford, Conn.). The dispersion was sonicated in consecutive 2 min bursts separated by a 1 min rest interval. The actual sonication time was 30 min. During sonication nitrogen was continuously passed over the dispersion. The soybean phosphatidylcholine (PL100) containing dispersions were processed at room temperature; to prevent chemical degradation an increase in temperature was prevented by cooling in ice-water. A temperature of 70 ^{o}C was chosen to process the dispersions with hydrogenated soybean phosphatidylcholine (PL100H). The phase transition temperature of pure PL100H vesicles in aqueous media is 51 ^{o}C. All dispersions were prepared in a 10 mM TRIS buffer, if necessary adjusted to pH 8.1 with diluted NaOH solution. To lower down the viscosity of the dispersions sometimes (as indicated) 0.8% NaCl was added. The dispersions were stored in the refrigerator.

The lipid compositions under investigation are shown in table 1.

Analytical Methods

Particle size was determined by dynamic light scattering with a Malvern 4600 apparatus (Malvern Ltd, Malvern, U.K.), equiped with a 25 mW helium/neon laser (NEC Corp., Tokyo, Japan). Using the Malvern PCS 2.4/2.3 software. Here with the method of cumulant expansion the mean diameter and the polydispersity of the dispersion was determined[9]; no correction for Mie scattering was made.

Zeta potentials were determined by measuring the mobilities of the liposomes in a cylindrical-cell microelectrophoresis apparatus (Mark II; Rank Brothers, Bottisham, U.K.) as described before[10].

The Microfluidizer™ was a type M110 apparatus (Microfluidics Corp., Newton, MA).

Table 1.

Composition	Molar Ratio
PL100	-
PL100/CHEMS	10/1
PL100/CHEMS/Chol	10/1/4
PL100/CHEMS/Chol	10/1/10
PL100H	-
PL100H/CHEMS	10/1
PL100H/CHEMS/Chol	10/1/4
PL100H/CHEMS/Chol	10/1/10

Composition of the bilayers of the prepared vesicles.

RESULTS

Particle Size and Size Distribution.

Different types of vesicles were prepared: vesicles with phospholipids forming a "fluid" (soybean phosphatidylcholine/PL100) or "gel" (hydrogenated soybean phosphatidylcholine/PL100) state bilayer (at temperatures below body temperature) with various amounts of charge inducing agent (CHEMS) and/or cholesterol.

In figure 1 the mean particle size is shown as a function of the number of passes through the interaction chamber for PL100H and PL100/CHEMS 10/1 vesicles. The PL100/CHEMS 10/1 profile is typical for all other liposome dispersions (not shown). Each data point is the average of readings from three separate measurements. The polydispersity index of the samples was dependent on the bilayer composition: after a few passes it reached values generally between 0.2 and 0.4. The first passage through the interaction chamber of the Microfluidizer™ reduced the mean diameter of the dispersions to a value below 0.25 μm. After the fifth pass the particle size alterations per cycle were relatively small compared to the initial changes. After 30 cycles the mean particle

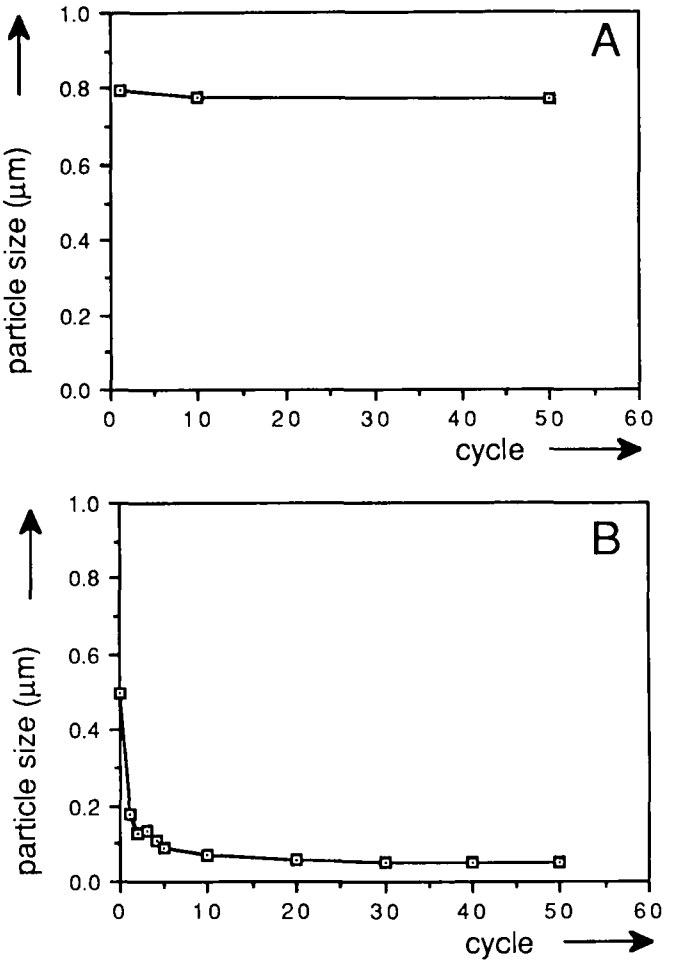

Figure 1. Mean particle size vs. number of passes.
a. PL100H. b. PL100/CHEMS:10/1

diameter reached a constant value between 0.05 and 0.20 µm for all but one composition. The only exception were pure PL100H vesicles; for this composition no particle size alterations could be achieved at all.

We tested two different types of interaction chambers, supplied with the Microfluidizer™, a type A and B chamber. The influence of the type of interaction chamber on the preparation process was limited. Chamber A gave a somewhat smaller particle size after the first few cycles, but after

Table 2.

Composition	Size (μm)	Polydisper-sity index
PL100	0.08	0.53
PL100/CHEMS:10/1	0.05	0.42
PL100/CHEMS/Chol:10/1/4	0.08	0.33
PL100/CHEMS/Chol:10/1/10	0.17	0.21
PL100H	0.77	0.82
PL100H/CHEMS:10/1	0.08	0.27
PL100H/CHEMS/Chol:10/1/4	0.07	0.27
PL100H/CHEMS/Chol:10/1/10	0.11	0.36

Particle size and polydispersity of liposome dispersions after 50 passes (chamber A).

50 passes the particle size and polydispersity were the same (results not shown).

In table 2 the mean particle size and polydispersity after 50 passes through interaction chamber A are shown.

In table 3 a comparison is made between the data for the minimum particle size and their corresponding polydispersity for the three different methods for sizing used in this study. As different types of size distributions were obtained with different methods the polydispersity data only can be evaluated per sizing technique (see also Discussion).

In general the PL100 containing vesicles are somewhat smaller than the corresponding vesicles containing PL100H. The size differences found for dispersions prepared by ultrasonication or the Microfluidizer™ were small. Increasing the cholesterol contents of the bilayers gave somewhat larger vesicles.

Zeta potentials

The zeta potentials of the dispersions are shown in table 4.

For the PL100 vesicles with CHEMS zeta potentials of -31/32 mV and for the PL100H vesicles with CHEMS zeta potentials of about -43 mV are

Table 3.

Composition	Prep. method	Size (μm)	P.D. index
PL100/CHEMS:10/1	EX	0.23	0.17
	US	0.04	0.29
	MF	0.05	0.42
PL100/CHEMS/Chol:10/1/4	EX	0.20	0.16
	US	0.05	0.21
	MF	0.08	0.33
PL100/CHEMS/Chol:10/1/10	EX	0.29	0.22
	US	0.08	0.58
	MF	0.17	0.40
PL100H/CHEMS:10/1	EX	0.27	0.26
	US	0.07	0.24
	MF	0.08	0.27
PL100H/CHEMS/Chol:10/1/4	EX	0.36	0.20
	US	0.09	0.21
	MF	0.07	0.27
PL100H/CHEMS/Chol:10/1/10	EX	0.36	0.25
	US	0.11	0.36
	MF	0.11	0.36

Sizes of liposomes in aqueous dispersions after sizing with different sizing techniques.
P.D. index=polydispersity index; EX=extrusion; US=ultrasonication; MF=Microfluidizer™.

found. As expected PL100H vesicles have no zeta potential at all. The zeta potential of the vesicles consisting of pure PL100 might be ascribed to the presence of the free fatty acids (See also discussion).

Recovery of phospholipid

The recovery of phospholipid in the dispersion after processing 50 ml of the dispersions was determined. The recovery was defined as the ratio between final (after processing) and initial phospholipid concentration. The mean size after processing is also presented it should be

Table 4.

Composition	zeta potential (mV)
PL100 before processing	-14
PL100 after 50 passes	-18
PL100/CHEMS:10/1	-32
PL100/CHEMS/Chol:10/1/4	-31
PL100/CHEMS/Chol:10/1/10	-32
PL100H before processing	0
PL100H after 50 passes	0
PL100H/CHEMS:10/1	-46
PL100H/CHEMS/Chol:10/1/4	-40
PL100H/CHEMS/Chol:10/1/10	-41

Zeta potentials of the vesicles in dispersion after processing and for PL100 and PL100H vesicles also before processing n=20 coefficient of variation=15%.

Table 5.

Comp.	Lipid conc. initial (μmol/ml)	Recovery x 100%	Particle size (μm)
a	100	73	0.11
a	200	57	0.08
a	300	48	0.08
a	400	43	0.21
b	100	25	0.17
b	200	46	0.13
b	300	53	0.19
b	400	48	0.20

Phospholipid recovery and size after processing different phospholipid concentrations. Composition: a. PL100/CHEMS/Chol:10/1/4 in 10 mM Tris pH=8.1 and 0.8% NaCl. b. PL100H/CHEMS/Chol:10/1/4 in 10 mM Tris pH=8.1 and 0.8% NaCl.

emphasized that the ionic strength of the dispersions in table 5 is much higher than in table 2. The average particle size for the dispersions with 0.8% NaCl tended to be larger than for similar liposome dispersions under low ionic strength conditions.

DISCUSSION

The Microfluidizer™ produced liposome dispersions with narrow size distributions. The mean vesicle diameters for the compositions under investigation ranged between 0.05 and 0.25 µm. These dispersions can be sterilized after homogenization by filtration through 0.2 µm membrane filters.

Particles with sizes below 100 nm might be preferred as drug carriers over larger vesicles, because of their tendency to prolonged circulation in the blood and their capacity to penetrate fenestrated endothelial barriers e.g. in the liver[11].

Comparison of the different production techniques shows that treatment with the Microfluidizer™ and the ultrasonication method gave, in general, similar mean vesicle sizes. The dispersions which were extruded through 0.2 µm filters had diameters of 0.2 µm or higher; in particular PL100 containing dispersions had a relatively low polydispersity index. In an earlier study we found that after extrusion the size distribution of the vesicles around the mean size was non-gausian[12]. We did not investigate whether the particle size distributions in the "microfluidized" or sonicated dispersions were gausian. As the shape of the size distribution curve has an impact on the mean particle size and polydispersity index, only dispersions exposed to the same sizing technique can be directly compared and interpreted. However, for similar size distributions and polydispersities, as found with the "microfluidized" and sonicated dispersions, the same behavior in vivo and vitro is expected.

An advantage of the Microfluidizer™ over sonication and extrusion is that higher lipid concentrations could be handled. Up to 400 µmol lipid/ml can be processed under favorable conditions. This implies a substantial

increase in encapsulation efficiency of the drug involved. If accidently the chamber is blocked by solid material the passage is easily cleaned by mounting the interaction chamber in the reverse direction and pumping the material out of the clogged system. The small increase of the zeta-potential after 50 cycles through the Microfluidizer™, suggested that some decomposition of the PL100 might take place during processing of the dispersion in the Microfluidizer™. PL100H vesicles did not show any signs of decomposition after processing.

The high viscosity of the highly concentrated dispersions was a limiting factor for a further increase in phospholipid concentration in order to maximize encapsulation efficiencies. In particular under low ionic strength conditions the dispersions cannot easily be manipulated above 120 µmol lipid per ml. Addition of 0.8% NaCl decreased the viscosity, but for concentrations above 250 µmol lipid/ml handling of the dispersions was still difficult and a substantial loss of lipids was observed even in the presence of 0.8% NaCl.

CONCLUSIONS

Liposome dispersions with rather well defined and narrow particle size distributions could be produced with the Microfluidizer™. When the dispersion did not reach the required size or size distribution in one production cycle the dispersion was recycled until the required size was reached. The apparatus can homogenize volumes down to about 40 ml in the recirculation mode. Since the manufacturer claims that also equipment for processing large volumes is available, the technique is in principle also suitable for the production of large volumes of liposomes. However, a potential problem for the use of the apparatus for production of liposomal dispersions for pharmaceutical purposes is that the dispersions contained small amounts of metal particles after production. Therefore some kind of sample clean up step has to be introduced in the production process after homogenization.

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REFERENCES

1. G. Gregoriades (ed.) "Liposome Technology Vol.I." CRC Press Inc., Boca Raton, FL, 1984.
2. D.J.A. Crommelin and G. Storm, in "Comprehensive Medicinal Chemistry. Vol.5." J.B. Taylor, ed, Pergamon Press Ltd, Oxford, 1988. (in press).
3. G. Storm, Thesis, University of Utrecht, 1987.
4. H. Talsma, H. Jousma, K. Nicolay and D.J.A. Crommelin, Int. J. of Pharm., 37, 171 (1987).
5. F. Szoka and D. Papahadjopoulos, Ann. Rev. Biophys. Bioeng., 9, 467 (1980).
6. E. Mayhew, J. King, R. Lazo, W.J.Vail and A.M. Green, Biochim. Biophys. Acta, 775, 169 (1984).
7. E. Mayhew, G.T. Nikolopoulos, J.J. King , A.A Siciliano, Pharm. Manufact., 2, 18 (1985).
8. E. Mayhew, S. Conroy, J. King, R. Lazo, G. Nikopoulos, A. Siciliano and W.J. Vail, in " Methods in Enzymology. Vol. 149. Drug and Enzyme Targeting. Part B.", R. Green, K.J. Widder, eds, Academic Press Inc, San Diego, 1987, p. 64.
9. J.C. Brown, P.N. Pusey and R. Dietz, J. Chem. Phys., 62, 1136 (1975).
10. D.J.A. Crommelin, J. Pharm. Sci., 73, 1559 (1984).
11. H.H. Spanjer, Thesis, University of Groningen, 1986.
12. H. Jousma, H. Talsma, F. Spies, J.G.H. Joosten, H.E. Junginger and D.J.A. Crommelin, Int. J. Pharm., 35, 263 (1978).