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LIPOSOME PREPARATION BY A NEW HIGH PRESSURE HOMOGENIZER GAULIN MICRON LAB 40

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ABSTRACT

High pressure expansion of lipid dispersions with a lab-scale homogenizer (Gaulin Micron LAB 40) was tested to produce forced lecithin hydration and subsequent liposome formation. A single-step liposome preparation method was developed. Lipid composition, pressure and the number of passages as the main process parameters were varied. Homogenizing with the new apparatus leads to small unilamellar vesicles (SUV),

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when 10% (w/w) lipid dispersions are used. Depending on the lipid composition the resulting vesicles ly from 25 up to 50 nm and show quite narrow particle size distribution. The cumulation of SUVs at their theoretical low size limits was detected after negative staining by transmission electron microscopy. Cryo-electron microscopy (CEM) of very thin vitrified layers of unfixed and unstained dispersions of vesicles confirmed in principle the negative staining data. Erosions of the especially stressed parts of the homogenizer, the valve, were monitored by scanning electron microscopy (SEM). They turned out, to be low, and this is particularly important for parenteral administration.

INTRODUCTION

Liposomes as a drug delivery system offer good chances for therapeutical advances especially in drug targeting and controlled release. They are at the time being introduced into industrial production. Ideal methods for large scale liposome production requiring economic batch sizes or continuous preparation as well as uniformity of the product and reproducibility of the process are mostly not yet realized and still in discussion.

Within the last two decades different lab-scale liposome preparation techniques have been developed. Recent reports describe high pressure homogenization [1,2,3,4].

High pressure expansion of lipid dispersions with a Gaulin Micron LAB 40 was used in this study. One of our aims was to determine size and dipersity of the

vesicles in correlation to pressure, number of passages and lipid composition. A further aim was to avoid the film method, the first step in many liposome production procedures, which cannot be scaled up without considerable difficulties.

MATERIALS AND METHODS

The lipids used for liposome preparation are summarized in the following Table.

High Pressure Homogenizer

Gaulin Micron LAB 40[D] is a homogenizer for sample volumes up to 40 ml. The apparatus is working according

TABLE 1: LIPIDS

Trade names	Abbrev.	species and purity
Epikuron 200[A]	E 200	soybean lecithin 92% phosphatidylcholine (PC)
Phosal 100[B]	P 100	soybean lecithin 90 - 96% PC
Phosal 80[B]	P 80	soybean lecithin 79 - 86% PC
Phosal 100 H I[B]	P 100HI	hydrogenated soybean lecithin, min. 94% PC
Phosal 100 H III[B]	P 100HIII	hydrogenated soybean lecithin, min. 87% PC
Cholesterol[C]	CH	

All other chemicals used were of analytical grade.

to the principle of high pressure expansion by passing a small and precise knife edge gap. An electronically controlled hydraulic valve actuator ensures reproducibility and pressure constantness. The actual pressure at the valve can be observed during the whole process. The pressure range reaches from 15 to 160 MPa. The following changes were required at the homogenizer: In order to start the experiments at defined temperatures a copper coil was mounted around the cylinder with the raw material (educt) and thermostated by a Haake F3-CH circulating water bath[E]. For the measurement of maximum heating rates the product was collected in a chamber made of PVC. This chamber was fitted exactly into the product cylinder and was intended to isolate the homogenized sample against rapid loss of heat.

Liposome Preparation

Two different methods were used to prepare samples for high pressure homogenization.

(1) The film method (handshaken method)[5] was used to prepare multilamellar vesicles (MLV) of distilled water or 0.9% (w/w) NaCl solution and lipid (10% w/w).

(2) Mixing dry lipids with water or buffer by gentle shaking was used to prepare raw lipid dispersions. All lipids not supplied in powder form were first rasped in the frozen state on a grater.

The dispersions (1) or (2) were homogenized by the Gaulin MICRON LAB 40 one to 50 times at pressures of 15 up to 140 MPa.

Analytical Methods

Cryo electron microscopy (CEM)

Thin vitrified layers of unfixed, unstained vesicle dispersions[6,7] were prepared by dipping perforated carbon coated copper grids[F] in 0.05 % (w/w) lipid

vesicle dispersions, removing most of the liquid by touching with blotting paper and allowing the grid to fall in liquid propane, cooled close to solidification temperature by liquid nitrogen. The grid was subsequently stored under liquid nitrogen and transfered into the cryochamber of an ultramicrotome (Reichert-Jung, Cambridge FC4)[G] for loading the cryocartrigde of the EM (Zeiss CEM 902)[H]. Processing temperature was always below 100 K. The cartridge was placed into the transfer unit (Zeiss cryotransfer system)[H] by means of a coupled pushrod and subsequently transfered to the CEM. EM was carried out under minimum dose conditions to keep electron beam damage as low as possible. Controls of the diffraction patterns showed, that the vitrified condition of the ice matrix of the specimen is maintained throughout the processing. For enhancing contrast micrographs were taken under large defocus conditions to take advantage of phase contrast. As an alternative electrons which suffered a loss of engergy of approximately 50 eV were used for imaging (electron spectroscopic imaging ESI). Recording was performed with the plate camera respectively an online connected image processing unit.

Freeze Etching

For freeze-etching, less than 1 microliter of the sample was dropped on a freshly cleaved mica rectangle of 3 x 10 mm. Afterwards a mica plate of the same size is placed on top with its long axis perpendicular to the first. The sandwich consisting of the two mica plates with the liposome suspension enclosed is instantaneously frozen by dipping into liquid propane under the same conditions as described above. This sample is used for fracturing in a device similarly constructed as reported by Nermut and Williams[8]. For

etching, the fractured suspension is kept in a freeze-etching unit (Balzers BAF 301)[I] at 172 K while a metal surface held at 122 K, working as a cold trap, is positioned 1 cm above for 30 s. Subsequently the etched surface was rotary shadowed by beam evaporators under 30° with approximately 20 Å platinum-carbon and for stabilising under 90° with about 300 Å carbon. The vacuum was better than 5 x 10^{-7} Torr. After thawing the platinum-carbon replica was floated off from the mica plate in common household bleach and cleaned from remaining lipid. The replica was then adsorbed on carbon coated grids for studying in the EM (Philips EM 400)[K].

Negative staining

Negatively stained samples were prepared by placing a drop of 1 microliter of the liposome suspension on a carbon coated grid. After waiting a minute to allow the liposomes adsorbing to the carbon film most of the liquid was removed by touching a blotting paper. Subsequently a drop of a 2 % (w/w) uranylacetate solution adjusted to pH 7 with NaOH was placed on the grid and removed after 1 min as described above. After drying the grids were examined in the electron microscope (Philips EM 400)[K].

Differential scanning calorimetry

Differential scanning calorimetry (DSC) by a Privalov DASM1[L] was used to determine the transition temperatures and enthalpies of the gel to liquid-crystalline phase transition of mixed P 100HI/CH (1:1, mol/mol) lipid bilayers[9]. 0.0188 mmolar lipid dispersions were scanned between 10 and 70°C.

TABLE 2: MEMBRANE FILTRATION AND SEDIMENTATION

	passing 220 nm filter	sedimentation
Epikuron 200[A]	yes	yes
Phosal 100[B]	yes	yes
Phosal 80[B]	yes	yes
Phosal 100 H I[B]	yes	no
Phosal 100 H III[B]	yes	gel

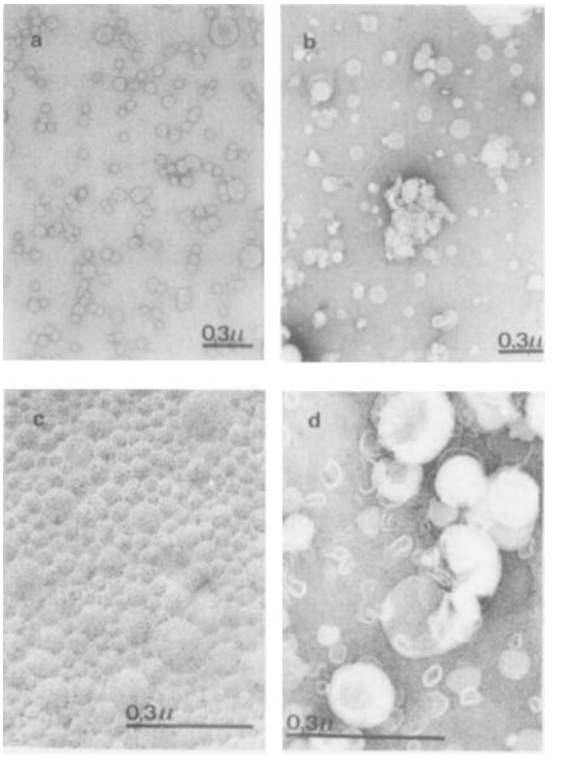

FIGURE 1:

Electron Micrographs of vesicle dispersions by negative staining (a, b, d) or freeze fracture (c)

RESULTS

Particle Size and Size Distribution

Hand shaken liposomes of different lipid composition in distilled water or isotonic NaCl solution were homogenized.

For preliminary characterization a membrane-filtration test was done immediately after preparation. Sedimentation effects were followed after at least one week storage of the filtered samples in sterile bottles. Results are shown in table 2.

Although all dispersions could be submitted to sterile filtration and no sedimentation was observed within hours, lateron alterations leading to sedimentation took place.

Samples at distinct steps of the preparation were visualized by negative staining in the electron microscope. In Figure 1 representative micrographs are shown. As can be seen, from P 100HI/CH-vesicles (Fig. 1a, 1x140 MPa; Fig. 1b 15x140 MPa) round or almost round images could be obtained, whereas E 200-vesicles (Fig. 1d, 10x15 MPa) seem to be somewhat damaged. On the micrographs diameters of about 300 vesicles were measured and size distributions were calculated. They are shown as histograms (Fig. 2 to Fig. 8). In addition five characteristic numbers of the distribution are performed. They are given in the legends of Figure 2 to Figure 8 and compared in the following Table.

The modus indicates the most often appearing vesicle diameter. The lower quartile, the median and the upper quartile are the diameters of the vesicles at 1/4, 1/2 and 3/4 of the distribution. A measure of the broadness of the distribution is the interquartile range, the distance in that half of the diameters fit.

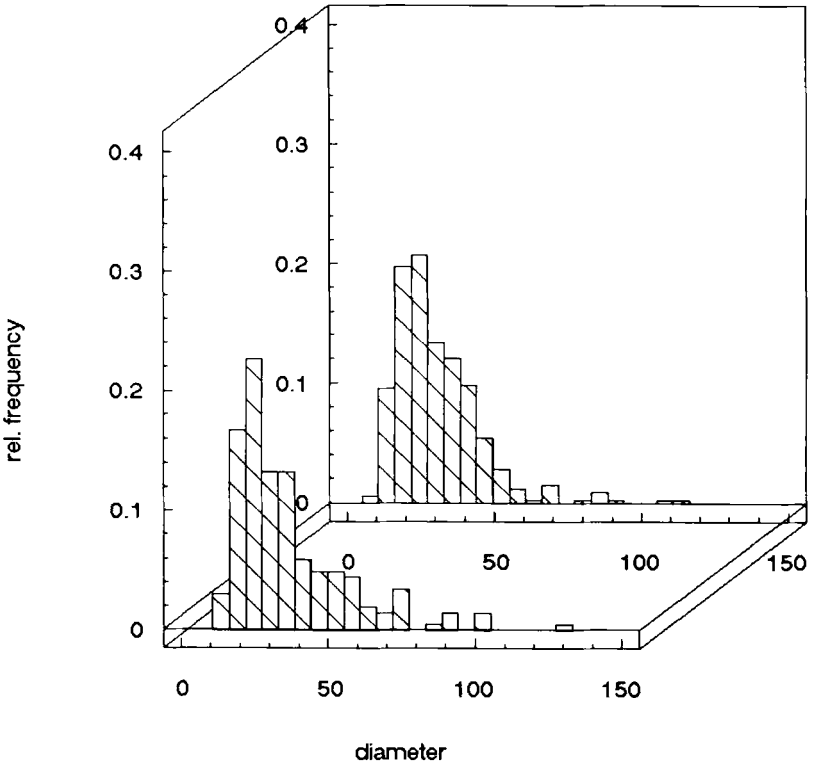

FIGURE 2

Size distribution of vesicles after 15 MPa homogenizing E 200 in dist. water (10 %, w/w)

Variable:	1 x 15 MPa	10 x 15 MPa
Sample size	203	313
Median	31 nm	28 nm
Mode	26 nm	24 nm
Lower quartile	24 nm	21 nm
Upper quartile	45 nm	38 nm
Interquartile range	21 nm	17 nm

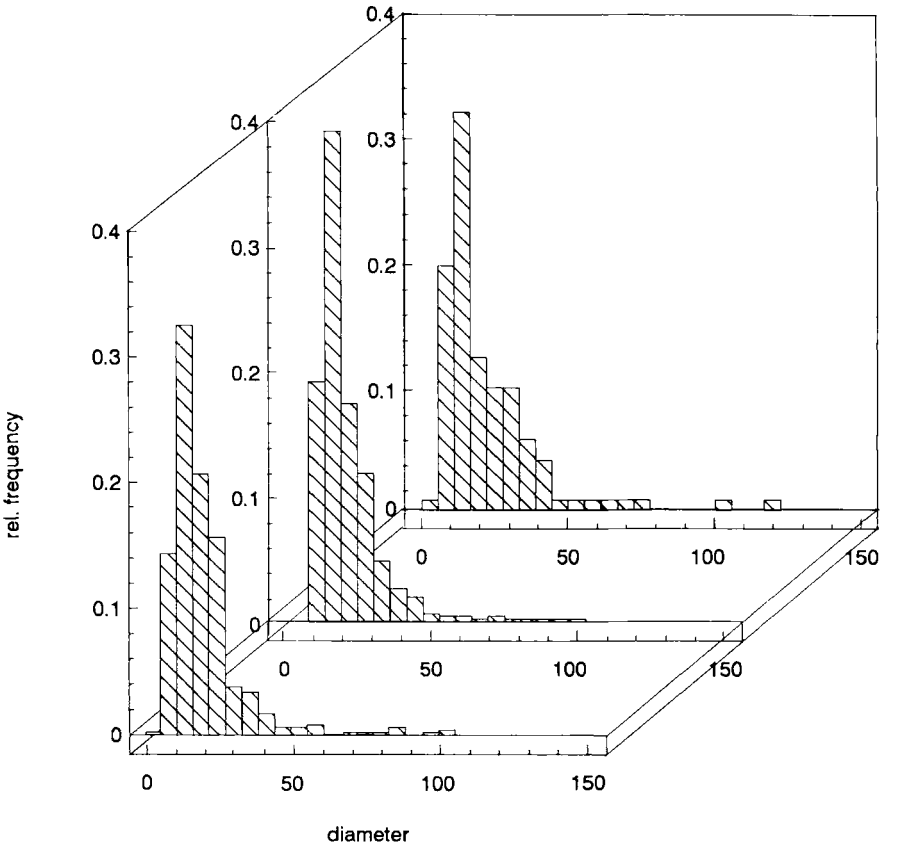

FIGURE 3

Size distribution of vesicles after 40 MPa homogenizing E 200 in dist. water (10 %, w/w)

Variable:	5 x 40 MPa	10 x 40 MPa	50 x 40 MPa
Sample size	354	307	303
Median	17 nm	17 nm	15 nm
Mode	12 nm	16 nm	12 nm
Lower quartile	13 nm	14 nm	12 nm
Upper quartile	24 nm	23 nm	27 nm
Interquartile range	11 nm	9 nm	15 nm

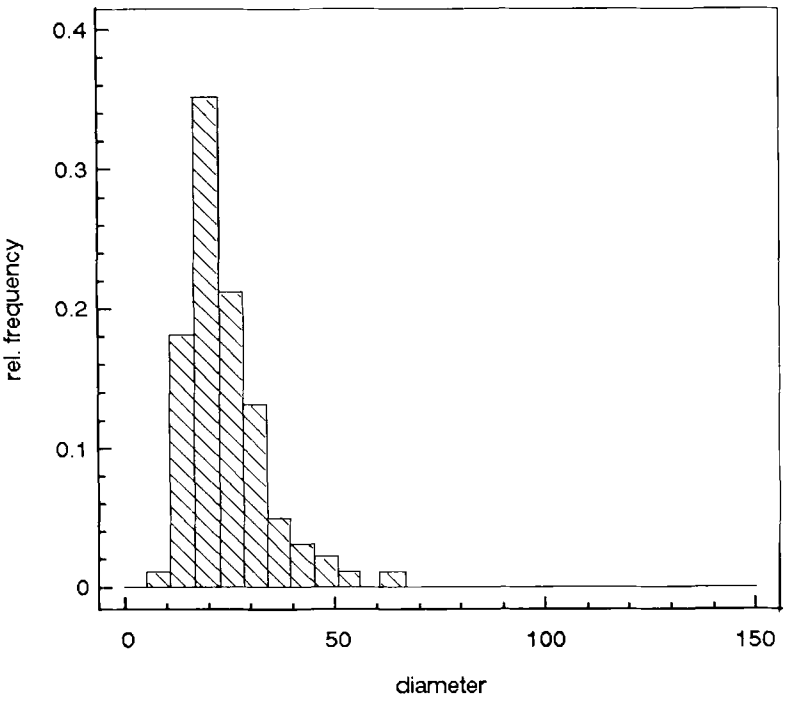

FIGURE 4

Size distribution of vesicles after 140 MPa homogenizing E 200 in dist. water (10 %, w/w)

Variable:	5 x 140 MPa
Sample size	312
Median	22 nm
Mode	16 nm
Lower quartile	18 nm
Upper quartile	28 nm
Interquartile range	10 nm

Several samples of vesicles were visualized by cryo electron microscopy. The micrographs are shown in Fig. 9. Pictures a, b, and c are from the plate camera , d from the image processing unit. As can be seen the holes of the carbon film are in part covered by vitrified water with vesicles therein. The vesicles can

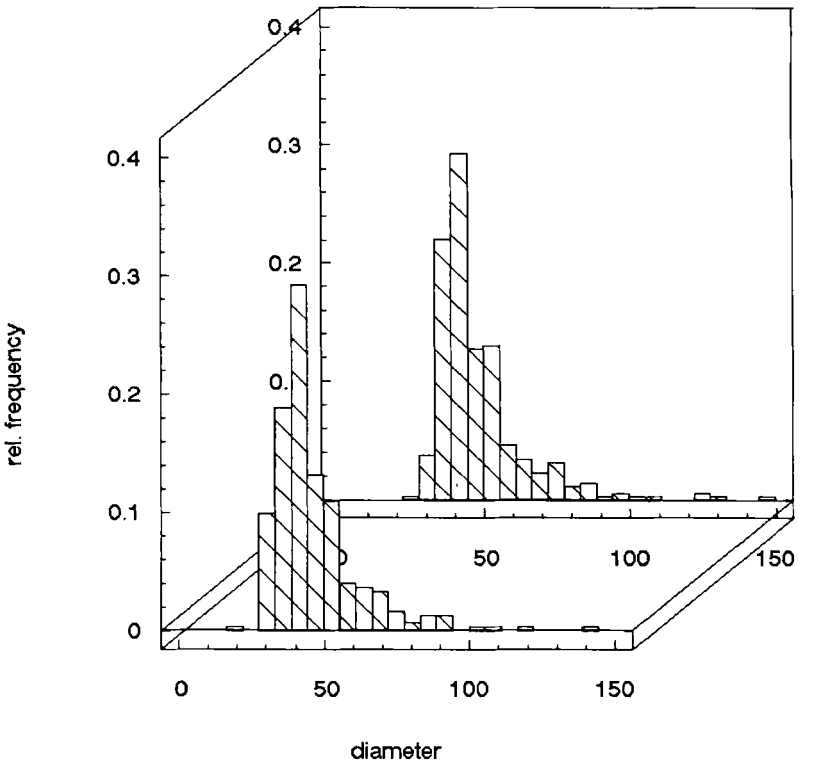

FIGURE 5

Size distribution of vesicles after 140 MPA homogenizing P 100 H I / CH (1:1, mol/mol) in dist. water (10%, w/w)

Variable:	1 x140 MPa	5 x 140 MPa
Sample size	301	349
Median	43 nm	43 nm
Mode	41 nm	39 nm
Lower quartile	37 nm	38 nm
Upper quartile	51 nm	52 nm
Interquartile range	13 nm	14 nm

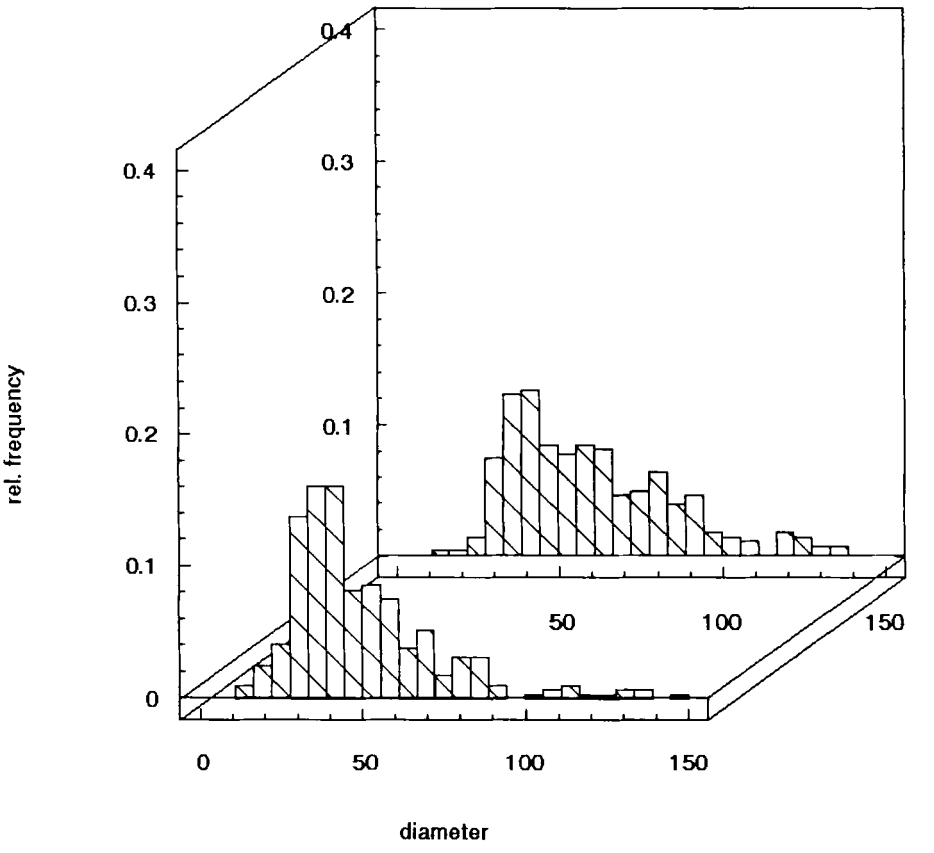

FIGURE 6

Size distribution of vesicles after 15 MPa homogenizing P100HI / CH (1:1, mol/mol) in 0.9% NaCl sol. (10%, w/w)

Variable:	1 x 15 MPa	5 x 15 MPa
Sample size	293	288
Median	43 nm	56 nm
Mode	41 nm	42 nm
Lower quartile	34 nm	41 nm
Upper quartile	59 nm	77 nm
Interquartile range	25 nm	37 nm

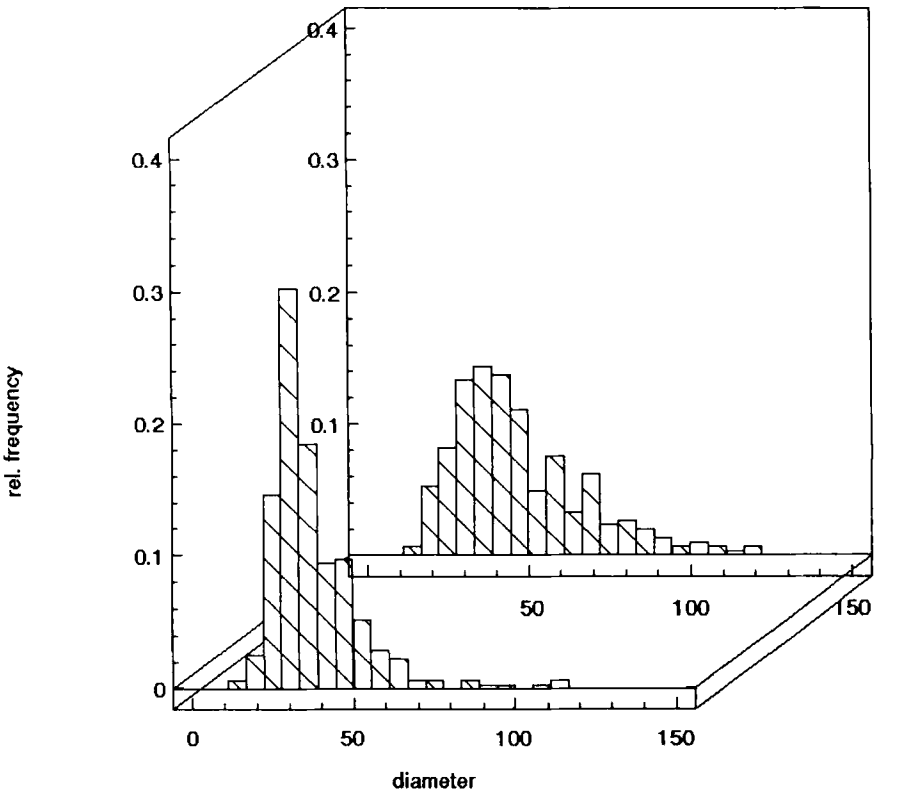

FIGURE 7

Size distribution of vesicles after 70 MPa homogenizing P100HI / CH (1:1, mol/mol) in 0.9% NaCl sol. (10%, w/w)

Variable:	1 x 70 MPa	10 x 70 MPa
Sample size	307	306
Median	34 nm	43 nm
Mode	31 nm	35 nm
Lower quartile	29 nm	33 nm
Upper quartile	43 nm	58 nm
Interquartile range	15 nm	25 nm

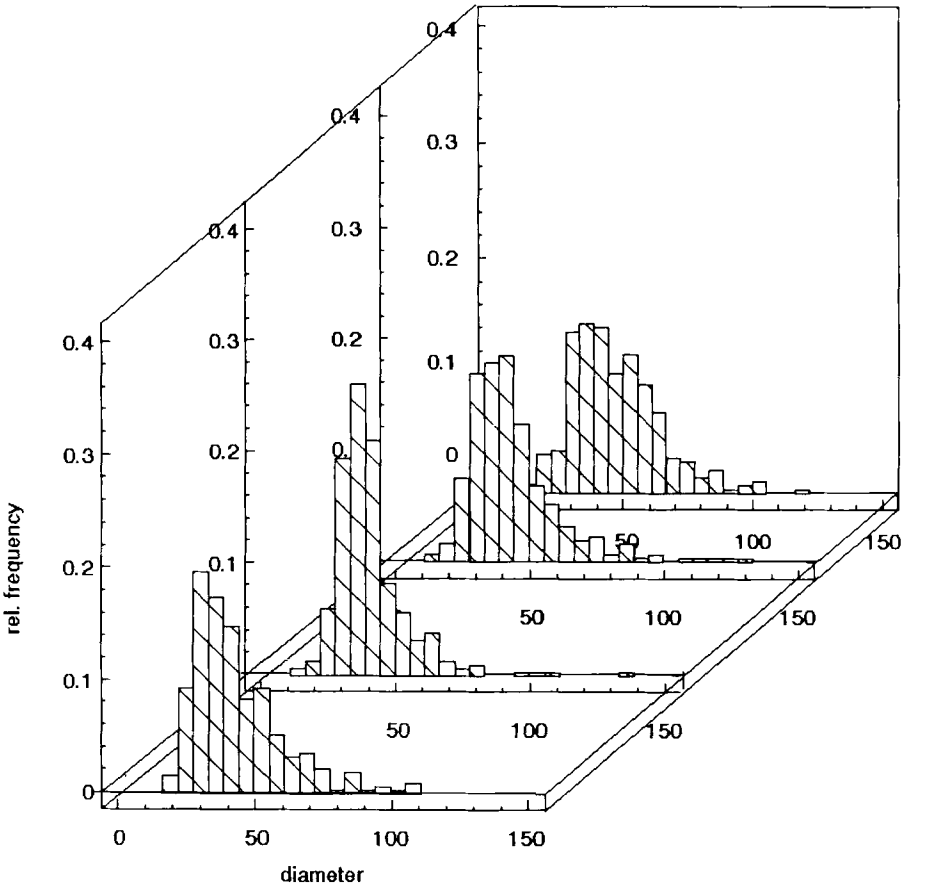

FIGURE 8

Size distribution of vesicles after 140 MPa homogenizing P100HI / CH (1:1, mol/mol) in 0.9% NaCl sol. (10%, w/w)

Variable:	1x140 MPa	5x140 MPa	10x140 MPa	15x140 MPa
Sample size	308	313	307	302
Median	39 nm	38 nm	40 nm	44 nm
Mode	33 nm	36 nm	34 nm	36 nm
Lower quartile	32 nm	32 nm	32 nm	34 nm
Upper quartile	52 nm	45 nm	50 nm	58 nm
Interquart. range	20 nm	12 nm	18 nm	26 nm

TABLE 3: LOWER/UPPER QUARTILE OF THE VESICLE DIAMETERS AFTER REPEATED HOMOGENIZATION AT VARYING PRESSURES

	E 200 dist. water			P 100HI/CH (1:1, mol/mol) dist.w. 0.9% NaCl sol.				
	15	40	140	140	15	70	140	MPa
1	24-45			37-51	34-59	29-43	32-52	
5		13-24	18-28	38-52	41-77		32-45	
10	21-38	14-23				33-58	32-50	
15							34-58	
50		12-27						

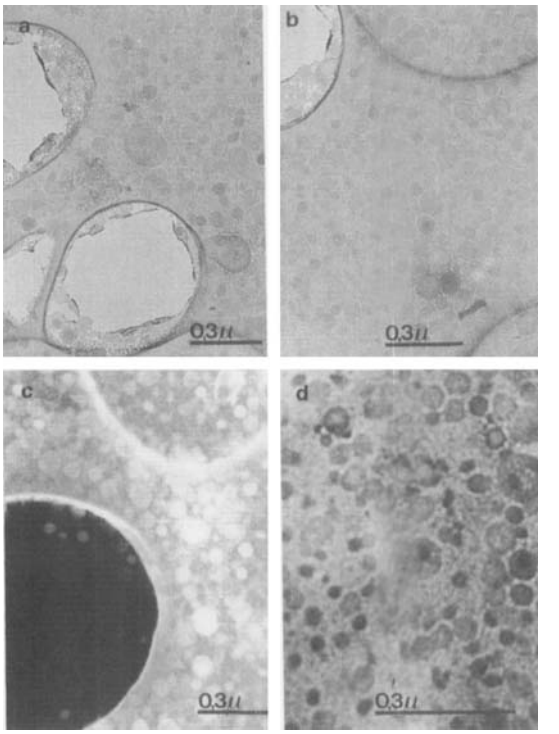

FIGURE 9:

Cryo Electron Micrographs of P100HI/CH-vesicles.

be imaged by ESI at an energy loss of 50 eV as carbon containing structures. The size distribution, determined from CEM compared with negative staining data showed very little discrepancy for P 100HI/CH-vesicles and confirmed in principle the results for E 200-vesicles.

A freeze fracture micrograph of P 100HI/CH-vesicles after 10 passages of 70 MPa is shown in Fig. 1c. There is no more than one bilayer visible.

Single Step Liposome Preparation

Lipid dispersions could be homogenized acccording to method (2) with lipid concentrations up to 100% (pure lipid). Below 60% (w/w) homogeneous vesicle dispersions were obtained during following dilution. In order to determine the homogeneity of mixed P 100HI/CH-bilayers, lecithin, cholesterol (50 mol%) and water were filled in the machine and repeatedly homogenized. Differential scanning calorimetry was used to follow, whether the phase transition peak disappears, as known from vesicle dispersions prepared by the film method and subsequent sonication. Fig.10 shows the differential scanning calorigramms.

Heat production, Abrasion

The maximum warming up of the homogenized dispersions is measured to calculate damages on thermolabile drugs e.g. proteins or peptides. Heat production is directly related with the pressure. The highest measured heating of water is 2.3 K / 10 MPa and of lipid dispersions (10% w/w) 2.6 K / 10 MPa. During regular operation the observed heating was much lower, as the product was recooled immediately.

The abrasion is monitored by scanning electron micrographs of the stressed parts of the valve. These

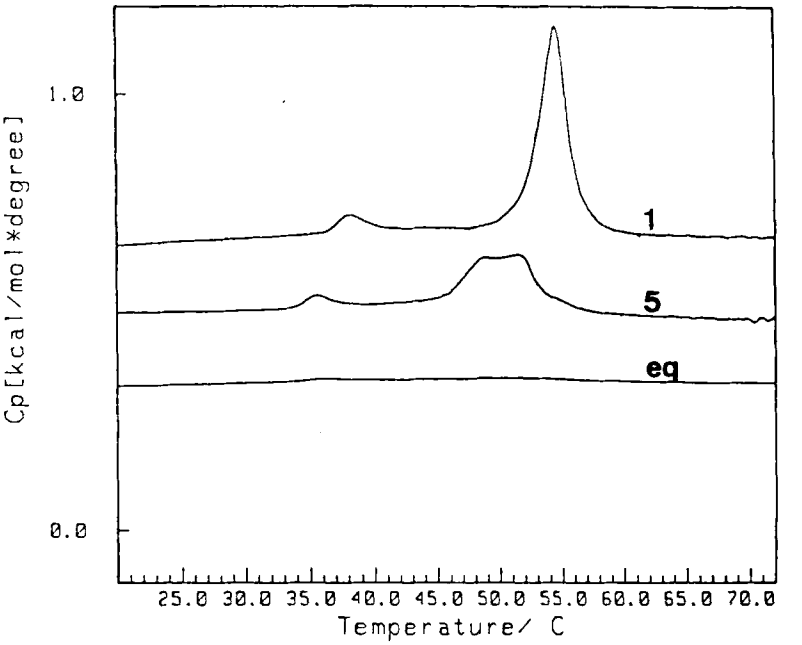

Figure 10:

Differential scanning calorigramm of an equimolar P 100HI / CH lipid dispersion after one (1) and five (5) homogenization cycles at 140 MPa and after 10 hours of equilibration (eq) above phase transition temperature in order to facilitate lateral diffusion[10]. Curve (1) and (5) are shifted by +0.25 respectively +0.5. The peak of the main transition gets smaller and finally disappears after repeated homogenizing and equilibration. The finally reached calorigramm fits exactly to that of vesicles of the same lipid composition, but prepared by the film method (not shown).

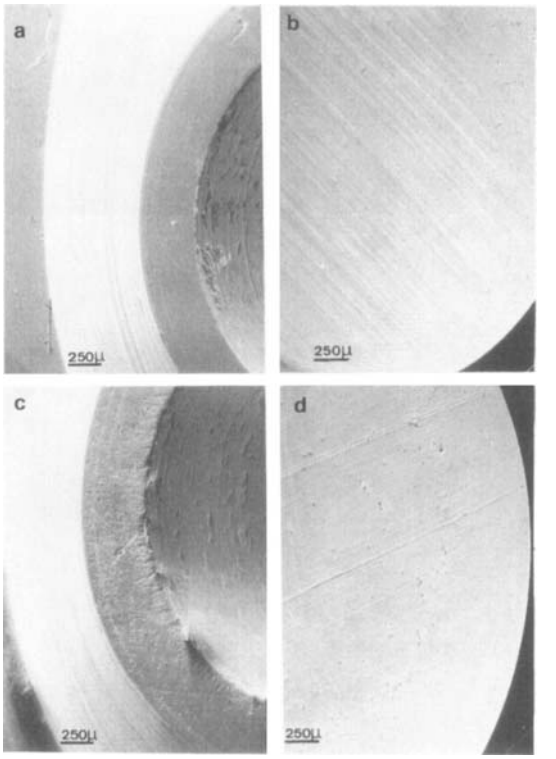

FIGURE 11:

Erosion of the Valve. Lower and upper part of a new valve (a, b) and of a 300-times used valve (c, d)

are the upper edge of the central bore of the valve and a ring-shaped area on the deflecting plate of the die. Fig. 11 shows the damage after more than 300 homogenization cycles at variing pressures. The difference between a new valve (Fig. 11a, b) and a used valve (Fig. 1 c, d) is visible at 30-fold magnification. At 100-fold magnification (Fig. 12a, b) details of erosion as chamfers on the edge and maximum

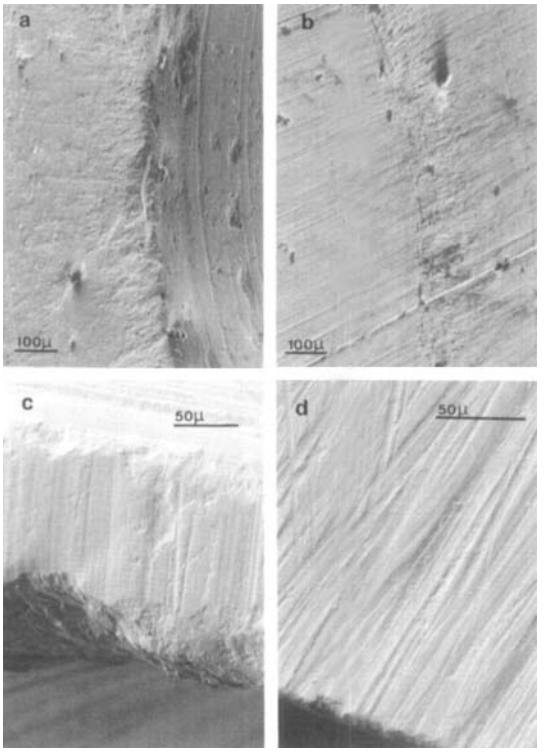

FIGURE 12:

Valve made of stainless steel (a, b) or ceramics (c, d)

five micron deep excavations at the previously smooth surface can be distinguished. An exact localization is given in Fig. 13. The used type of ceramics as an alternative material for highly stressed tools shows beside the described defects splintering damages in some ten micron scale (Fig. 12c, d).

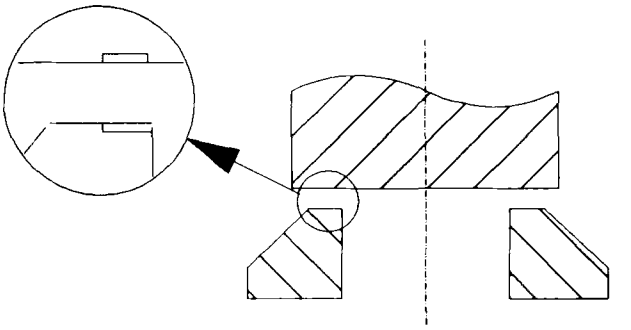

FIGURE 13:

Profile of the Valve

The zone of maximum erosion lies on both sides at the inner half of the radial knife edge gap. This zone could be postulated as zone of cavitation.

DISCUSSION

Size reduction of the vesicles

The main homogenization mechanisms are known to be[11,12,13]:

(1) cavitation

(2) shearing during laminar and turbulent flow

According to Bernoulli's law the static pressure in a fluid is decreased at high velocity of flow. If the local pressure is falling below the steam pressure, bubbles filled with steam or gas arise and grow until reelevation of the pressure causes their implosion. The surrounding water and lipid bilayers are accelerated towards the middle of the bubble followed by shock waves. This effects high local stress, e.g. taering into parts of the bilayer and excavation of the steel surface. Compare Fig. 13.

The main results of this stress are the reduction of the numbers of bilayers and decreasing diameters of the vesicles. The sizes and particle size disributions

of the vesicles were found to vary with the number of passages and the pressure used during repeated homogenization. A great portion of the vesicles, produced by the homogenizer show the smallest possible diameters already after the first passage at lowest used pressures (15 Mpa). A few more passings lead to increased uniformity by enlarging the fraction of vesicles showing smaller diameters. The general tendency is smaller vesicle sizes and more narrow distributions with rising pressures. During repeated homogenization the distribution reaches an optimum after a distinct number of passages. If further homogenization cycles are performed the distribution is broadened again. The smallest reached vesicle diameter was found to correspond with the lipid mixture. As expected from the theory of Israellachvili[14] vesicles below the critical radius are not stable. This is the reason for the observed non-gaussian distribution or more detailed for the absence of smaller particles. Mixed P 100HI/CH-vesicles show more expanded minimum diameters as E 200-vesicles. The ratio of the polar head group to the hydrocarbon volume and critical chain length, the critical packing parameter, is smaller for fluid chain lecithin (E 200) than for saturated frozen chain lecithin (P 100HI) and cholesterol.

Homogeneity

The depression of the phase transition enthalpy (Fig. 10) could be interpreted as an indicator of increasing homogeneity of the lecithin/cholesterol mixture in the bilayer. The regions of less than 50% (mol/mol) cholesterol in the bilayer, which still show gel to liquid-crystalline phase transition disappear. If convenient dispersion of both lipids is achieved, homogeneity is completed by lateral diffusion within

hours. Mixed lecithin/cholesterol bilayer vesicles are known to show asymmetric distribution[15] of the cholesterol between the inner and the outer layer, so in our case also flip-flop and intervesicle exchange phenomena should take place during homogenization and equilibration.

CONCLUSIONS

Dispersions of nearly uniform small unilamellar vesicles could be produced with the MICRON LAB 40. With the energy expended electron microscopic techniqes a detailed insight in the process of vesicle formation during high pressure homogenization could be gained. The process parameters pressure and number of passages were optimized. As the apparatus is able to homogenize lipids without prior hydration and fully homogeneous dispersions of lipid mixtures are reached, a real one-step liposome production process could be achieved. Since the manufacturer claims, that process data of this lab-scale homogenizer can be transferred without changes to the production scale machines, a new tool for research and development in the liposome field is now available. Advantageous in comparison with other homogenizers is the constantness and permanent control of the pressure and the relatively low erosion of the valve made of stainless steel. The ceramic valve however could not reach this good quality; the search for better suitable material must go on. The examination of possible damages for lipids and drugs especially proteins and peptides resulted in first hopeful experiences with insulin[16], but further studies will be necessary.

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