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Factors involved in the production of liposomes with a high-pressure homogenizer

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Abstract

A variety of factors were evaluated in order to establish the characteristics of the liposomes obtained with a high-pressure homogenizer (Microfluidizer 110S). The experimental mean diameter of the liposomes and the width of their size distribution were correlated to surfaces calculated from the responses obtained by the combination of two groups of factors. First, the inlet pressure of the homogenizer, the times that the samples were processed (cycles) and the bulk ionic strength. Second, the phospholipid (P) and ethanol concentrations. The variation of the entrapped liposome volume upon the pressure and cycles was also studied. All the calculated surfaces are statistically significant and have a low S.E. of estimate. Mean liposome diameter decreases with increasing inlet pressure, number of cycles and ethanol concentration, and increases raising ionic strength. No P concentration effect was observed. The variation of the entrapped volume of liposomes upon the cycles and pressure has a similar behavior to the diameter. Within the studied variable range, microfluidization does not increase the P oxidation index, nor does it significantly alter the P concentration.

Keywords: Experimental design; High-pressure homogenization; Liposome; Microfluidizer; Phospholipid; Statistical optimization

1. Introduction

After liposomes were described in the 1960's a great number of scientific applications were predicted based on their structure. Nowadays they are commonly used as model membranes, but on the other hand their use as drug delivery system has a gradual, progressive application. In recent years, several lipid-based drug formulations have been commercialized and some others are undergoing advanced clinical studies (Lasic, 1998). Even the most questioned route of administration, dermal delivery, which has resulted in a number of contradictory results over years (Schreier and Bouwstra, 1994) is being clarified; interactions between liposomes and human skin have been demonstrated (Kuijk-Meuwissen et al., 1998; Kir-

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javainen et al., 1999) and allowed to elucidate the action mechanism.

High-pressure homogenizers allow the pharmaceutical industry to obtain different formulations, from emulsions and suspensions to nanoparticles (Lamprecht et al., 1999) and so, they are frequently employed for the large-scale production of liposomes (Talsma et al., 1989; Brandl et al., 1990; Bachmann et al., 1993; Lasic, 1993; Brandl et al., 1998). The Microfluidizer is one of several commercially available homogenizers that can be directly scaled-up in order to obtain a large-scale production of liposomes, thereby avoiding intermediate studies. While it was patent pending, it was first used to obtain liposomes (Mayhew et al., 1984) and, from that time on, the literature has described several cases that also applied it to the preparation of liposomes. There is, however, no exhaustive characterization of the factors that affect the process. As the characteristics of the liposome suspensions clearly determine their encapsulation efficiency and application (Kulkarni et al., 1995), an accurate knowledge of the homogenization procedure should facilitate the choice of the work conditions for obtaining a predetermined type of liposomes.

In the present study we have systematically investigated some variables involved in the production of liposomes with the Microfluidizer 110S. Depending on the device, factors such as the inlet pressure and the number of times that a sample is processed have been evaluated, as have external variables, such as ionic strength and phospholipid (P) concentration. As a consequence of the described effects of ethanol on the phosphatidylcholine bilayers caused by the association of the alcohol with the membranes (Löbbecke and Cevc, 1995; Trandum et al., 1999), the ethanol concentration has also been taken into account as a factor.

For this research, in order to minimize the number of experiments, a statistical optimization strategy has been applied using factorial designs. This procedure has proved to be a very useful tool for studying multifactor response surfaces (Deming and Morgan, 1987; Hassan et al., 1992).

2. Materials and methods

2.1. Materials

Pro-lipo S was purchased from Lucas Meyer S.A. This raw material is a mixture of soybean Ps (20% molar negatively charged), ethanol, glycerol and water, 30:20:10:40 w/w/w/w. Emulmetik 930 (composition over ca. 95% w/w soybean phosphatidylcholine (SPC), maximum 3% w/w of lysophosphatidylcholine, maximum 1% w/w of other Ps and maximum 3% w/w of moisture or residual oil content) was supplied by Lucas Meyer S.A. PD-10 columns (Sephadex G-25 medium, 1.5×5 cm) were purchased from Amersham Pharmacia Biotech. All other chemicals were of analytical grade.

2.2. Preparation of liposomes

Suspensions constituted by a mixture of multi and oligolamellar charged liposomes (Leigh, 1985; Perret et al., 1991) were obtained by pouring Pro-lipo S into aqueous medium at room temperature and mixing. Neutral liposomes were obtained by magnetic stirring of Emulmetik 930 with aqueous medium at 50–60°C. The standard aqueous medium used was sodium phosphate buffer 10 mM, pH 7.4. When needed, the ionic strength of the medium was adjusted with NaCl.

The initial liposome suspensions were processed with a high-pressure homogenizer (Microfluidizer 110S, Newton, USA) in the non-recirculation mode. The inlet pressure of the homogenizer (homogenization pressure is 1273 times inlet presure) and the number of times that the samples were processed (cycles) were adjusted for each batch in accordance with the established experimental design. In the case of Emulmetik 930 suspensions, the required amount of ethanol was poured just before the homogenization. The minimum volume of the samples processed was 30 ml. All homogenizations were carried out at room temperature and, therefore, above the transition temperature of the liposome bilayers, as all the liposome membranes are made from natural (unsaturated) soybean Ps.

2.3. Measurement of the liposome size

The size of the liposomes was measured using a Microtrac Ultrafine Particle Analyser 150 spectrometer (UPA). This device measures vesicle size distribution by dynamic light scattering operating with heterodyne detection (Trainer et al., 1992; Ostrowsky, 1993). The only mathematical modelling of the raw data assumes that Brownian motion originates velocity distribution of the particles. Particle size distributions are calculated directly from the power spectrum by fast Fourier transform and, consequently, are not obtained using any other mathematical restriction than that indicated previously. This is why the analysis performed with the UPA does not include any parameter to estimate the accuracy of measured size distribution (such as the polydispersity index in photon correlation spectroscopy devices).

The UPA is equipped with a diode laser with a wavelength of 780 nm and a nominal output of 3 mW of optical power. The time of data acquisition was 10 min. The analyses were carried out at least 1 h after the preparation of the processed suspensions. Samples were diluted with their aqueous medium in order to obtain a satisfactory signal in the detector.

As logarithmic normal size distributions of liposomes were usually obtained, the results are presented as the mean diameter and width (half the central range of the measured particle size distribution enclosing 68% of the vesicles) of the volume (or weight) distribution (Bachmann et al., 1993). In the results, both previous data are shown with their respective experimental S.Ds.

2.4. Determination of the entrapped volume of the liposomes

The entrapped volume was calculated using buffer with $Na_2Cr_2O_7$ at a concentration of 1 mM as water-soluble marker (Richards and Gardner, 1978; New, 1990). Due to the presence of alcohols in the Pro-lipo S suspensions, the chromate stability was checked in an aqueous medium containing ethanol and glycerol at the same concentration as a liposome suspension at a P concentration of 50 mg ml^{-1}. No change in Cr^{6+} concentration was detected in 1 month of storage at 4°C.

The $Na_2Cr_2O_7$ was added to the buffer when the suspensions were prepared. The non-encapsulated molecules were separated from liposomes by size exclusion chromatography using PD-10 columns pre-saturated with Ps (New, 1990) and operating at gravity flux. In order to minimize the osmotic change of the vesicles, the fluent medium used was the aqueous medium of the samples containing Na_2SO_4 2 mM. The initial and the eluate-chromate concentrations were determined by atomic absorption using a graphite chamber. The quantifications were carried out at least 1 h after the preparation of the processed suspensions.

2.5. Determination of the P oxidation

The detection of conjugated dienes was employed to monitor P chemical stability during homogenization. Sample aliquots were diluted in absolute ethanol at a P concentration of 0.4 mg ml^{-1}, and the absorbance measured at 233 and 215 nm (Klein, 1970). The ratio between the two values increases with the amount of conjugate dienes in the P fatty acid chains and detects the occurrence of radical chain reactions. Accordingly, with the lower detection limit in the technique assumed by Klein ($A_{233}/A_{215} = 0.001$) and with the molar absorption coefficient for dienes indicated by New (30 000 M^{-1} cm^{-1}), the minimum increase detectable in our samples is 1.25 mmol diene mol^{-1} P.

During measurement, the reference cuvet was filled with a solution of absolute ethanol and aqueous sample buffer in the same ratio as the sample cuvet. There are few authors who take this procedure into account, but it has to be undertaken in this way in order to subtract the slight turbidity caused by the buffer salts when the samples are diluted in ethanol (Lang and Vigo-Pelfrey, 1993).

2.6. Quantification of the P concentration

The P concentration was determined by two methods. In suspensions obtained without chromate, it was calculated using the spectrophotometric method based on the complexation of Ps

with ammonium ferrothiocyanate (Steward, 1980).

When chromate was added to the preparations in order to calculate the entrapped volume of the liposomes, interference was detected. In controls, the calculated P concentrations were 84% of the actual concentrations. Consequently, in such experiments, the P concentration was calculated by measuring the apparent absorption at 550 nm. At this wavelength, there is no absorbance of the chromate ion, and only the apparent absorption caused by the liposomes is detected. For each sample processed with the Microfluidizer, a calibration curve of the P concentration versus absorbance was obtained by dilution of aliquots with the buffer. After realizing size exclusion chromatography, the aliquots of the liposomal fraction were conveniently diluted and their absorbance used to calculate the P concentration using their respective calibration curve. This method can be used only when there is no variation in the liposome size distribution, a phenomena that does not occur during the size-exclusion chromatography taken with Sepadex G-25 medium (PD-10 columns) due to the fact that the fractionation range of this type of columns causes liposomes to be eluted in the void volume. As control, no variation in size distribution was detected by laser light scattering between samples with liposomes of 500 or 150 nm and their respective 5 consecutive liposome eluted fractions of 0.2 ml. These results agree with the literature, as agarose (Sepharose) or polyacrylamide (Sephacryl) columns of about 40 cm in length and operating at low flow rates (~ 5 ml h^{-1}) are required to achieve a partial fractionation of liposomes in size distribution and stability studies (Wong et al., 1982; Schurtenberger and Hauser, 1984; McConnell and Schullery, 1985; Perevucnik et al., 1985; Lesieur et al., 1993).

2.7. Experimental design and data analysis

In order to find the relationship between the factors selected and the characteristics of liposomes prepared by high-pressure homogenization, response surface regression procedures were used to correlate the responses. In all cases the number of experiments and their factor combinations allowed the measured responses to be fitted by stepwise method to initial lineal polynomial models using coded factors (Deming and Morgan, 1987). Due to this, the final equations are simplified models that contain only significant parameters ($P > 0.05$). Replicates were performed to calculate the S.E. of estimate. All results were evaluated by analysis of the variance (ANOVA).

The size of the liposomes was studied as a function of two groups of factors. The first group is formed by three factors, the number of cycles that the samples are processed with the homogenizer (ranging from 1 to 9); the inlet pressure of the homogenizer (ranging from 1 to 4 bar); and the ionic strength of the bulk medium (ranging from 22 to 155 mM). The initial liposome suspensions were obtained with Pro-lipo S at a concentration of 50 mg P g^{-1}. In this case, in order to evaluate the three variables with a limited set of experiments, level combinations were chosen in the so-called central composite experimental design, with three replicates in all star levels. As a means of approximating the region of multifactor response, the initial equation used was a full second-order polynomial (FSP) model. This equation includes constant, first-order, second-order and interaction terms and is one of the most useful models. As an example, all combinations are shown in Table 1.

The second group of factors is formed by the P concentration (ranging from 10 to 40 mg g^{-1}) and by the ethanol concentration (ranging from 0 to 160 mg g^{-1}). The relative concentration of the components was chosen within the range that allows the formation of liposomes in the ternary system formed by the ethanol, the Ps and the aqueous medium (Perret et al., 1991). The initial liposome suspensions were obtained from Emulmetik 930 at a concentration of 160 mg P g^{-1}. Before processing the samples with the Microfluidizer (2 cycles, 2.4 bar), the necessary amounts of ethanol were added to obtain the required level combinations of the factors. The initial equation used in the fitting was an FSP model.

On the other hand, the entrapped volume was studied only as a function of the number of cycles

and the inlet pressure of the homogenizer. The ionic strength was fixed at 22 mM and the P concentration at 50 mg g^{-1} (obtained from Prolipo S). To minimize the number of experiments a central composite experimental design was applied also using an FSP model to obtain the surface response.

The experimental design used for studying the P recovery and oxidation was a star design, which implies a simpler combination levels than the central composite design used in the previous experiments. The factors selected to determine P recovery were the cycles, the pressure and the initial P suspension concentration. Only the cycles and pressure were varied in checking the P oxidation during the microfluidization.

3. Results and discussion

3.1. Study of P concentration changes during sample processing

The combination levels between the initial P concentration of the samples, the cycles and the inlet pressure are shown in Table 2. Results, expressed as percentage of the initial concentration value, show that the final P concentration of the samples is practically independent from all the previous factors. Although from the Table 2 a mean value of the recovery a lower than 100% is obtained ($97.6 \pm 3.40\%$), it is within the relative experimental error of the quantification method employed ($\sim 5\%$). These high recoveries of P in the processed samples allow the obtention of the suspensions with practically no loss of raw material. These results contrast with those indicated by Talsma et al. (1989) who, working with MLVs obtained by the film method at lipid concentrations equal and higher than 100 μmol ml^{-1}, found a maximum and minimum recoveries of 75 and 25%. These recoveries were dependent on the P composition and on the initial P concentration.

The initial concentration of the samples and methods of preparation of the suspensions could cause such differences. In our case, the maximum concentration employed was 100 mg P ml^{-1} and the initial suspensions were prepared from Prolipo S, a system that in very few minutes, with aqueous medium and under stirring, forms liposomes without the presence of coarse P particles. Consequently, when the initial samples are treated with the homogenizer, all the Ps pass through the narrow nipples and through the interaction chamber and is finally obtained at the discharge tube.

Table 1
Factor combinations (coded values in brackets), replicates and responses of the multifactorial study for the size of the liposomes

Cycles	Pressure (bar)	Ionic strength (mM)	*n*	Diameter (nm)	Width (nm)
0	0	88	3	647 ± 125	221 ± 77.4
3 (−1)	0.8 (−1)	55 (−1)	1	231	276
3 (−1)	0.8 (−1)	121 (1)	1	320	325
3 (−1)	2.4 (1)	55 (−1)	1	141	139
3 (−1)	2.4 (1)	121 (1)	1	176	167
7 (1)	1.6 (−1)	55 (−1)	1	153	150
7 (1)	1.6 (−1)	121 (1)	1	219	215
7 (1)	3.2 (1)	55 (−1)	1	91.7	63
7 (1)	3.2 (1)	121 (1)	1	109	98
1 (−2)	2.4 (0)	88 (0)	3	350 ± 6.8	377 ± 25.2
5 (0)	0.8 (−2)	88 (0)	3	336 ± 16.8	431 ± 19.2
5 (0)	2.4 (0)	22 (−2)	3	127 ± 4.6	121 ± 10.7
5 (0)	2.4 (0)	88 (0)	3	160 ± 1.5	138 ± 0.6
5 (0)	2.4 (0)	155 (2)	3	179 ± 4.7	141 ± 12.1
5 (0)	4 (2)	88 (0)	3	104 ± 19.4	74.0 ± 17.8
9 (2)	2.4 (0)	88 (0)	3	134 ± 13.1	133 ± 25.4

Table 2
Recovery of P and oxidation indexes for the processed samples as function of the factor combinations levels (coded values in brackets)

Initial P concentration (mg ml^{-1})	Cycles	Pressure (bar)	n	Phosphilipid recovery (%)	Oxidation index (% of initial)
10 (−0.8)	5 (0)	2.4 (0)	1	103.2	–
100 (1)	5 (0)	2.4 (0)	1	95.1	–
50 (0)	5 (0)	0.8 (−1)	2	90.4/100	100/101
50 (0)	5 (0)	4 (1)	2	99.0/96.1	99.4/100
50 (0)	5 (0)	2.4 (0)	1	99.8	100
50 (0)	9 (1)	2.4 (0)	1	93.2	99.7
50 (0)	1 (−1)	2.4 (0)	1	99.0	100
Mean				97.6 ± 3.40	100 ± 0.33

3.2. Effect of microfluidification on the chemical stability of the Ps

The mean oxidation index of freshly and non-processed liposome suspensions made from Prolipo S was 0.25 ± 0.04. The processed sample indexes are shown in Table 2 expressed as a percentage of the initial value of the non-processed samples. As no increase of the oxidation index was detected, results suggest that, in the ranges studied, the homogenizer does not promote the radical reactions that, in the absence of oxygen, lead to the formation of conjugated dienes in the fatty acid chains of the Ps.

3.3. Effect of cycles, pressure and ionic strength on the liposome size

Table 1 shows the mean vesicle diameter, the width, and their respective experimental S.Ds. obtained from the analysis of the samples treated with the Microfluidizer 110S. As reference, the first row indicates the results for a group of non-processed suspensions. Results indicate that even in the minimum conditions of pressure, the first pass through the interaction chamber causes an important variation of the size of the liposomes. Fig. 1a–d show the response surfaces of the processed samples describing the relationship between mean diameter and width of liposome size distributions as functions of cycles, pressure and ionic strength. These functions are given in Eq. (1) Eq. (2), respectively

$$\text{Diameter (nm)} = 151 - 49.3\left(\frac{C-5}{2}\right) - 55.7\left(\frac{p-2.4}{0.8}\right) + 15.8\left(\frac{IS-88}{33}\right) + 21.9\left(\frac{C-5}{2}\right)^2 + 16.3\left(\frac{p-2.4}{0.8}\right)^2 \quad (1)$$

$$\text{Width (nm)} = 129 - 57.7\left(\frac{C-5}{2}\right) - 82.6\left(\frac{p-2.4}{0.8}\right) + 9.34\left(\frac{IS-88}{33}\right) + 30.7\left(\frac{C-5}{2}\right)^2 + 30.1\left(\frac{p-2.4}{0.8}\right)^2 \quad (2)$$

where C indicates the number of cycles, p the inlet pressure and IS the ionic strength of the aqueous medium. The expressions between brackets show the coding transformations used with the factors, and allow using the fitted parameters with the absolute value of the factors. The results of the ANOVA analysis of the models are shown in Table 3. Both models have good coefficients of multiple determination and pass the statistical test for the effectiveness of the factors ($F_{calculed} > F_{tabulated}$). On the other hand, the S.Es of estimate values for the models have no practical significance, i.e. the error is very low compared with the response value and with the accuracy of the methods employed in obtaining liposomes.

Populations observed in samples processed at

high pressure and high number of cycles were mainly unimodal, the other being bimodal suspensions with different ratios between both populations. However, examining the evolution of the size distributions for the results (the ratio between the liposome populations), it was possible to predetermine the conditions in order to obtain two different unimodal samples at an ionic strength of 22 mM. The first was obtained by processing the samples ($n = 3$) 9 cycles at 4 bar, and the vesicle population obtained was 39 ± 7 nm in mean diameter, with 15 ± 4 nm in width for distribution size. Liposomes of the second type of unimodal samples ($n = 3$), processed 1 cycle at 2 bar, were 319 ± 6 nm in diameter and 83.2 ± 13.4 nm in width. Taking S.E. of estimate (18.6 nm; Table 3) and experimental variability into account, the measured mean diameters of the unimodal samples are comparable with those obtained by Eq. (1), i.e. 62 and 353 nm respectively. The opposite is true for the case of width, as the expected values are 73 and 407 nm correspondingly (S.E. of estimate, 25.0 nm), considerably different from the experimental values. The fact that mean diameter is a measure of central tendency could explain these results, as, during the observed progressive diminution of liposome size due to homogenization, the mean diameter will also decrease concomitantly, and no local minimum of the mean diameter will be possible. Therefore, as observed, the experimental and the expected mean diameters would show a good correlation in the case of both uni- and bimodal populations. On the other hand, the width evaluates how greatly

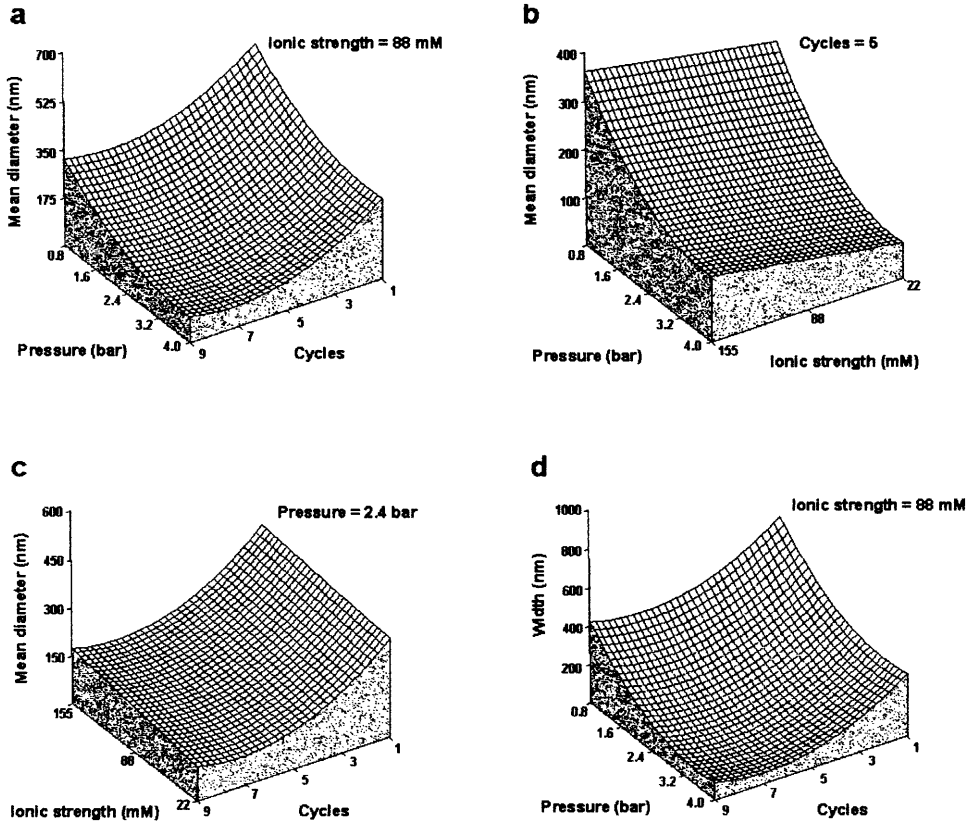

Fig. 1. The diameter and the width of the liposome size distributions vary with the operating conditions of Microfluidizer 110S and the ionic strength of the bulk medium. The response surfaces were calculated from the Eq. (1) and Eq. (2). In each graphic, the value of the constant factor is shown.

Table 3
ANOVA results for response surfaces

Equation	Equation	R^2	F_{calc} ($F_{critical}$) $\alpha = 0.05$	S.E. of estimate
(1)	Diameter (nm) = f(*C*,*p*,*IS*)	0.982	125 (2.64)	18.6
(2)	Width (nm) = f(*C*,*p*,*IS*)	0.983	130 (2.64)	25.0
(3)	Diameter (nm) = f(*E*)	0.762	86 (4.21)	102
(4)	Width (nm) = f(*E*)	0.658	52 (4.21)	164
(5)	Entrapped volume (l mol^{-1}) = f(*C*,*p*)	0.889	80 (2.61)	0.122

the size distribution is spread and, therefore, it is only dependent on the dispersion of the analyzed samples. As the surface width response has been obtained from unimodal and bimodal samples, any interpolation inside the bimodal region will reflect the heterogeneity of the samples used to obtain Eq. (2). This is the case for samples processed 1 cycle at 2 bar. Their slight dispersion caused by their unimodal character is a local minimum not included in the calculated surface of response and, consequently, the experimental width is considerably different from its estimated value. In the case of samples processed 9 cycles at 4 bar, the difference between both values is not so high. This phenomenon could be explained by the fact that the samples employed to calculate the surface at comparable pressure and number of cycles similar to the unimodal samples, are already mainly unimodal.

The effect of the number of cycles and the inlet pressure on the liposome diameter is shown in Fig. 1a for case of a suspension with an ionic strength of 88 mM. Although the slope of the diameter curves tends to zero at high pressures, any increase of the pressure causes a decreasing diameter in all the studied cycle range. For any number of cycles, minimum liposome size is obtained by processing samples at an inlet pressure of 4 bar. These results show that in the conditions of the present study, the intensity of the high shear forces, the turbulence and/or the cavitation produced during the homogenization process determine liposome size. Diameter also diminishes by increasing the number of cycles, but not in the same way as with pressure. It shows a saturation-like behavior i.e. at a given pressure, any increase in the number of cycles larger than a critical value will not decrease the mean diameter of the liposomes. Consequently, the times the sample is treated through the interaction chamber only has an important effect in the initial steps. This steady value for the liposome size is reached for all pressures approximately at 7 cycles. Results are congruent with the previous findings of other authors working with several high-pressure homogenizers. Talsma et al. (1989) reported similar behavior when working with liposomes constituted by different mixtures of SPC or hydrogenated SPC with cholesterol and cholesterylhemisuccinate. Conversely, in the case of liposomes constituted by mixtures of natural or hydrogenated SPC with cholesterol, Brandl et al. (1990) and Bachmann et al. (1993) observed, with two different high-pressure homogenizers, that increasing the number of cycles beyond a certain pressure limit resulted in (re-)increasing liposome diameter. In those studies, and inside the analyzed range of cycles and pressures, this phenomenon was not observed when liposomes were comprised only of SPC.

In accordance with Brandl et al. (1990), the cycle saturation-like behavior observed in the present work could be explained in terms of an initial formation of P structures below the lowest limit of possible liposome size (about 20 nm) during homogenization. The authors referred above suggest that these highly unstable structures would rapidly fuse with one another, producing larger liposomes for any number of cycles greater than a critical value. Another explanation is also possible by taking into account the fact that, first, our results show a critical number of cycles in the pressure range studied, including the low pressures that produced liposomes with a diameter considerably different from the lowest limit and, second, in the range studied, it is possible, for any number of cycles, to diminish lipo-

some size by increasing the pressure, without any (re-)increasing diameter effect being observed. Consequently, the cycle saturation-like effect could be described by stating that, beyond the cycles' critical value, and for a determinate liposome diameter distribution, the forces involved in the vesicle disruption inside the interaction chamber are not intense enough to down-size the liposomes. This effect has been described also for the case of the mechanical degradation of polymers induced by the microfluidification (Silvestri and Gabrielson, 1991; Silvestri et al., 1991; Cencia-Rohan and Silvestri, 1993). In these cases apparent mechanical degradation rate constants can be calculated and their value establish the relationship between the reciprocal polymer molecular weights and the times that the samples are processed. The constants appear to be dependent on the chemical nature of the polymers as well as the interaction chamber pressure, and the process has a limiting average weight to which the polymer may be mechanically degraded. As the origin of this down-sizing is the exposure of the polymers covalent bonds to mechanical stresses, a parallelism can be established with the microfluidification stress caused on the hydrophobic forces involved in the integrity of the liposome bilayer.

The relationship between pressure, ionic strength and liposome mean diameter is shown in Fig. 1b, represented in the case of samples processed 5 cycles. Any increase in the ionic strength yields an increment in the vesicle diameter. This behavior is also observed with the method based on the controlled dilution of organic solvents (Isele et al., 1994) when NaCl concentration ranges from 0 to 78 mM. This effect can be related to the use of a raw material with charged Ps. As the salt concentration increases, the screening between the charged bilayers also rises and, in consequence, diminishes the electrostatic repulsion between them, promoting the formation of large vesicles. A similar relationship is observed in Fig. 1c where ionic strength, cycles and diameter are represented when pressure is maintained constant at 2,4 bar.

The width has a similar behavior to mean diameter. Fig. 1d shows the surface response when pressure and cycles are varied at a constant ionic strength of 88 mM. The dependence of the width on the pressure and the cycles shows that, excluding the local minimum of the unimodal sample processed 1 cycle at 2 bar (the width rises between 1 and 2 cycles), the treatment with the homogenizer has the effect of narrowing down the particle size distribution at the same time as the diameter decreases.

For all the representations shown in Fig. 1, it has to be taken into account that stacked surfaces are obtained when the non-represented factors varies. For example, in the case of Fig. 1a, an increase of 33 mM in ionic strength will result in a new surface displaced upward 9.30 nm.

The previous results show that an accurate control of the factors involved in the homogenization procedure predetermines the diameter and width of the liposome size distribution. Obviously, in the case of using another initial liposome suspension, the surface responses will have different values than previously shown, but a similar behavior can be expected.

3.4. Effect of P concentration and ethanol concentration on the liposome size

The diameter and width models were calculated from the experimental responses and are given in Eq. (3) Eq. (4):

$$\text{Diameter (nm)} = 288 - 118\left(\frac{E-80}{40}\right) \quad (3)$$

$$\text{Width (nm)} = 276 - 147\left(\frac{E-80}{40}\right) \quad (4)$$

where E is the ethanol concentration in mg g^{-1}.

The results of the ANOVA analysis (Table 3) show a significant correlation of the factor. The responses (Fig. 2) indicate that the size of the liposomes obtained in 2 cycles at 2.4 bar has a linear dependence upon the ethanol concentration of the bulk medium, the higher the ethanol concentration, the lower the diameter and width of vesicle size distribution. These results are not in the same line as those observed by Pons et al. (1993) for the case of the ethanol injection method. In such experiments, the liposome diameter decreases with the P concentration working at

constant final ethanol concentration. Contrarily, in our study no dependence of the vesicle diameter upon the P concentration is observed in the studied P range. Therefore, the preparation method is a variable that critically determines the contribution of the P concentration to the vesicle size. On the other hand, as has been previously demonstrated (Komatsu and Okada, 1995a,b), ethanol has no effect on vesicle size or on bilayer permeability when it is simply mixed with liposomes obtained with Ps not forming interdigitated structures; but it induces the massive aggregation and/or fusion of small unilamellar vesicles of dipalmitoylphosphatidylcholine (McConnell and Schullery, 1985; Komatsu et al., 1993). Hence, our results suggest that, if, as we propose in the range studied, there is no formation of P structures below the lowest limit of possible liposome size, ethanol concentration increases the disruption capability of high-pressure homogenizers on the liposomes comprised of SPC, allowing the obtention of smaller vesicles in fewer cycles or at a smaller pressure. Otherwise, taking into account the hypothesis of Brandl et al. (1990), the ethanol effect could be explained in terms of a reduction of the tendency of very small vesicles to fuse into bigger vesicles.

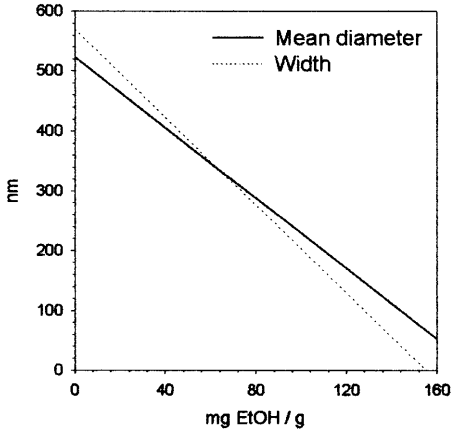

Fig. 2. The graphic shows the important effect of the ethanol (EtOH) concentration on the physical properties of the liposomes obtained at 2.4 bar and 2 cycles. No P concentration effect was detected between 10 and 40 mg g^{-1}.

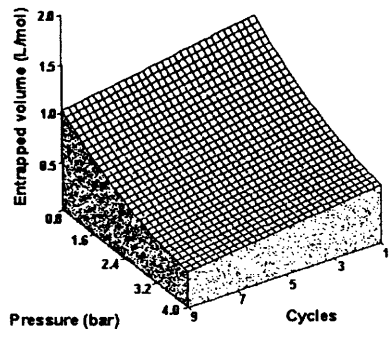

Fig. 3. The entrapped volume of the liposomes obtained with the Microfluidizer 110S is a function of the pressure and the times that the samples are processed. The ionic strength was fixed at 22 mM.

3.5. Effect of cycles and pressure on the entrapped volume

As in the previous results, a good surface response is obtained (Table 3 and Fig. 3) in this case in accordance with the expression 5.

$$\text{Entrapped volume (l mol}^{-1}) = 0.722 - 0.0716\left(\frac{C-5}{2}\right) - 0.180\left(\frac{p-2.4}{0.8}\right) + 0.0305\left(\frac{p-2.4}{0.8}\right)^2 + 0.0191\left(\frac{C-5}{2}\right)\left(\frac{p-2.4}{0.8}\right) \quad (5)$$

The entrapped volume has a similar mathematical dependence as the vesicle mean diameter due to the effect of the aqueous internal volume being a function of the vesicle diameter and lamellarity. However, maximum relative error is larger than in all the size fits. For the smaller liposomes (39 nm), it is almost a 30% of the estimated value (0.421 l mol^{-1}).

4. Conclusions

The control of pressure, the number of cycles, the bulk ionic strength and the ethanol concentration in the production of liposomes with a high-pressure homogenizer makes the process controllable. From Pro-lipo S, two different unimodal liposome suspensions can be obtained by

adjusting the levels of these factors and so, together with P recovery and preservation, makes this an optimum method for large-scale production.

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