Liposomes Prepared by High-Pressure Homogenizers

By Ramon Barnadas i Rodríguez and Manuel Sabés i Xamaní

Introduction

High-pressure homogenizers are used for the preparation of liposomes and lipid dispersions because of their vesicle disruption capability.1–7 In general, the initial suspension to be processed is composed of multilamellar liposomes that will be downsized inside the device by applying a large amount of energy to the suspension. The sample is injected at high and constant pressure in a specially designed part of the homogenizer where the rearrangement of liposome structure takes place due to turbulence, cavitation, and/or shear phenomena. The characteristics of the section where homogenization occurs represent one of the main differences between the models provided by the principal manufacturers.8 Some have a dynamic valve, whereas others have a fixed geometry, and still others are equipped with dynamic or static homogenizing valves. In all cases, the maximum process pressures reached by these instruments (normally about 30,000 lb/in², but, in special cases, up to 60,000 lb/in²) allow them to homogenize samples with a phospholipid concentration higher than 150 mg/ml. Another advantage associated with these devices is that, in most cases, small models have their corresponding scaled-up homogenizers for large-scale production and, subsequently, results can be transferred directly from laboratories to industry.

Properties of liposomes prepared by high-pressure homogenization depend on a first set of parameters related to the homogenizer and a
second group of factors associated with the sample. In the case of a given device with a specific homogenizing piece, the pressure and number of times that the sample is processed clearly determine the size distribution of the liposomes obtained. Sample factors include aspects such as phospholipid composition and concentration, initial size distribution and lamellarity of the liposomes, temperature, and composition and ionic strength of the bulk medium. In this chapter we describe procedures for obtaining liposomes and proteoliposomes (with a membrane protein), using a high-pressure homogenizer with a homogenizing piece having no moving parts, and the effect of some of the factors that influence vesicle size distribution.

Materials and General Procedures

Phospholipid Sources

All soybean phospholipids are purchased from Lucas Meyer (Hamburg, Germany). Emulmetik 930 is a deoiled, phosphatidylcholine-enriched fraction of soybean lecithin. It contains a minimum of 97% phospholipids, mainly phosphatidylcholine [minimum, 72% (w/w)], phosphatidylethanolamine [minimum, 8% (w/w)], phosphatidylinositol (maximum, 1%), and lysophosphatidylcholine [maximum, 3% (w/w)]. Emulmetik 950 is a hydrogenated soybean lecithin. It contains hydrogenated phosphatidylcholine [minimum, 95% (w/w)], lysophosphatidylcholine [maximum, 1% (w/w)], other phospholipids [maximum, 2.5% (w/w)], and oil [maximum, 1.0% (w/w)]. Pro-Lipo-S is a mixture of phosphatidylcholine and other soybean phospholipids (30%, w/w) as well as a hydrophilic medium (water, ethanol, and glycerol). This mixture mainly forms stacked, negatively charged bilayers that, when mixed with aqueous medium by stirring at room temperature, convert into liposomes. Egg yolk phosphatidylcholine is purified according to the method described by Singleton et al.10

Purification of Bacteriorhodopsin

Purple membrane containing bacteriorhodopsin is obtained from Halo-
bacterium salinarum as described by Oesterhelt and Stoeckenius.11 The membrane sheets isolated have a lipid-to-protein ratio of 1:3 (w/w).

9 S. Leigh, European patent application, application number 85301602.0; publication number, 0 158 441 (1985).
High-Pressure Homogenization

A Microfluidizer 110S (Microfluidics, Newton, MA) is utilized to prepare liposomes by high-pressure homogenization. In this laboratory-scale model, homogenization pressure is 230 times the inlet pressure. This device is equipped with a ceramic interaction chamber with fixed geometry where homogenization takes place. When the sample is processed inside, the flow splits into two main streams. They are forced to impact with one another at great velocity before leaving the interaction chamber. Depending on the characteristics of the sample and of the pressure, this recombination results in a specific reduction of the size of the vesicles present in the suspension. When working with slurried and/or concentrated suspensions at low pressures, the interaction chamber can become plugged. In this case, the chamber can be cleared easily by reversing its position in order to back flush. As a result of the position of a spool valve, the Microfluidizer 110S can operate by recirculating the processed sample to a product inlet reservoir or in a nonrecirculating mode. A removable coil and bath allow, when necessary, control of the temperature of the sample immediately before processing inside the interaction chamber.

Determination of Liposome Size

The size distribution of liposomes is measured by dynamic light scattering, using an ultrafine particle analyzer (UPA) 150 spectrometer (Microtrac, Montgomeryville, PA). This device operates by means of heterodyne detection\(^\text{12,13}\) and, for mathematical modeling, it assumes that only Brownian motion produces the velocity distribution of the particles. The spectrometer is equipped with a diode laser having a wavelength of 780 nm, and has an optical power of 3 mW. Analysis acquisition time is 10 min, and the samples are diluted with their aqueous medium to obtain a satisfactory signal in the detector. Results are presented as volume (or mass) distribution and are expressed as the mean diameter and width (half the central range of the measured particle size distribution that contains 68% of the vesicles).

Liposome Homogenization in Nonrecirculation Mode

Principle

Because of the constant pressure applied to the sample, large-scale preparation of liposomes with high-pressure homogenizers is highly reproducible. Therefore, at a given pressure, liposome size distribution

depends on the number of times that vesicles pass through the interaction chamber, and on the characteristics of their own suspension.\textsuperscript{14} When a non-recirculating mode of operation is selected, all the suspension undergo the same process, and, consequently, the times (number of cycles) that all the liposomes are processed clearly determine the final diameter of the vesicles. On the other hand, as the bilayer charge influences liposome size during their formation,\textsuperscript{15,16} the ionic strength of the bulk medium is an important parameter to control, in order to regulate the bilayer potential and, as a result, vesicle size distribution.

\textit{Methods}

To obtain the initial liposome raw suspension, sodium phosphate buffer (10 mM, pH 7.4) is poured into and mixed with Pro-Lipo-S (the Steward assay can be employed\textsuperscript{17} in order to determine the final phospholipid concentration, thus avoiding buffer interference), or, alternatively, it is possible to use a non-phosphate-containing buffer (e.g., HEPES). The ionic strength (IS) of the aqueous medium is adjusted with required quantities of NaCl and the phospholipid concentration is kept constant in all samples and equal to 50 mg/ml. All homogenizations are carried out at room temperature. To study the effect of cycles ($C$, ranging from 1 to 9), inlet pressure ($p$, ranging from 0.8 to 4 atm), and ionic strength (ranging from 22 to 155 mM) on liposome size distribution, a central composite experimental design\textsuperscript{18} is used. The combination and independent replicate factors of this design are shown in Table I. After processing the samples under the desired conditions, the size distribution of vesicles is obtained with the UPA 150. The relationship between the factors ($C$, $p$, and IS) and the responses (mean diameter and width) is calculated by the stepwise method, fitting empirical, full second-order polynomial models that include constant, first-order, second-order, and interaction terms. In these equations, the factor levels are expressed in coded values ranging from $-C_0^2$ to 2. This procedure allows the estimated values of the empirical parameters not to depend on each other and to facilitate the matrix manipulations.\textsuperscript{18} Consequently, the general expression of the equations is

Response = constant + $\alpha_1 C^* + \alpha_2 p^* + \alpha_3 IS^* + \beta_1 C^{*2} + \beta_2 p^{*2} + \beta_3 IS^{*2} + \gamma_1 C^* p^* + \gamma_2 C^* IS^* + \gamma_3 p^* IS^*$ \hfill (1)

where $\alpha_1$, $\beta_1$, and $\gamma_1$ are the empirical parameters calculated by the step-wise method, and

$$C^* = \frac{C - 5}{2}$$ \hfill (2)

$$p^* = \frac{p - 2.4}{0.8}$$ \hfill (3)

$$IS^* = \frac{IS - 88}{33}$$ \hfill (4)

After correlations are realized, the final equations contain only the significant parameters.

## TABLE I

**Experimental Design Used to Study Non-recirculating Homogenization**

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Inlet pressure</th>
<th>Ionic strength</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute (atm)</td>
<td>Coded</td>
</tr>
<tr>
<td>1</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>-2</td>
</tr>
<tr>
<td>5</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2</td>
</tr>
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<td>5</td>
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<td>3</td>
<td>1.6</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>-1</td>
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<tr>
<td>3</td>
<td>3.2</td>
<td>1</td>
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<tr>
<td>3</td>
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<td>1</td>
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<td>7</td>
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<td>7</td>
<td>1.6</td>
<td>-1</td>
</tr>
<tr>
<td>7</td>
<td>3.2</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>3.2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Factor combinations and replicates of the experimental design used to study the effect of pressure, cycles, and ionic strength on liposome size distribution obtained with the Microfluidizer 110S. Factor levels are indicated in absolute and coded values. Reprinted from *International Journal of Pharmaceutics*, 213, R. Barnadas and Manuel Sabés, “Factors involved in the production of liposomes with a high-pressure homogenizer,” 175–186 (2001), with permission from Elsevier Inc.*
Results

The fitted empirical equations obtained [Eqs. (5) and (6)] are shown in Table II. Both models pass the statistical test for the effectiveness of the factors, have good coefficients of multiple correlation, and have low standard errors of estimate. The slopes of the surface responses are significantly dependent on the three factors. Some examples of surface responses are shown in Fig. 1. In the studied range, pressure has a continuous effect on liposome size (Fig. 1A and B) as any increase in pressure causes a decrease in liposome diameter, although at high pressures the slope of mean diameter and width curves tend to zero. In the case of cycles, however, any increment in the number of cycles larger than approximately 7 does not significantly decrease the mean diameter or the width. Although not as strong as pressure and cycles, ionic strength also has an appreciable effect on liposome size distribution (Fig. 1C and D). As a result of the screening of bilayer electrical charges and, therefore, a diminution of the interbilayer repulsion, any increase in ionic strength causes an increase in liposome size.

From the point of view of the modality of the liposome suspensions, homogenization of Pro-Lipo-S yields mainly bimodal populations of vesicles. But from the surface responses and from the size distribution results, it is possible to predetermine the necessary conditions for obtaining two different unimodal samples. First, by processing samples for 9 cycles at 4 atm of inlet pressure and an ionic strength of 22 mM, small vesicles are obtained with a mean diameter of 39 7 and 15 4 nm in width (n = 3). From Eq. (5) and Eq. (6) in Table II, the estimated values obtained with the previous factor levels are, correspondingly, 62 and 73 nm. Conditions to obtain the second unimodal suspension are attained by taking into account the evolution of the ratio between the liposome

<table>
<thead>
<tr>
<th>Equation no.</th>
<th>Response</th>
<th>$R^2$</th>
<th>$F_{\text{calc}}$ ($F_{\text{crit}} = 2.64$)</th>
<th>Standard error of estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eq. (5)</td>
<td>Mean diameter (nm) = 151 – 49.3 $C^<em>$ – 55.7$p^</em>$ + 15.8$I^*$ + 21.9 $C^{*2}$ + 16.3$p^{*2}$</td>
<td>0.982</td>
<td>125</td>
<td>18.6</td>
</tr>
<tr>
<td>Eq. (6)</td>
<td>Width (nm) = 129 – 57.7 $C^<em>$ – 82.6$p^</em>$ + 9.34$I^*$ + 30.7 $C^{*2}$ + 30.1$p^{*2}$</td>
<td>0.983</td>
<td>130</td>
<td>25.0</td>
</tr>
</tbody>
</table>

*Fitted empirical equations of mean diameter and width and their statistical results. The studied factors are the inlet pressure of the Microfluidizer, the number of cycles, and the ionic strength of the bulk medium.*

TABLE II

Effect of Pressure, Cycles, and Ionic Strength on Liposome Size Distribution

<table>
<thead>
<tr>
<th>Equation no.</th>
<th>Response</th>
<th>$R^2$</th>
<th>$F_{\text{calc}}$ ($F_{\text{crit}} = 2.64$)</th>
<th>Standard error of estimate</th>
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<td>0.983</td>
<td>130</td>
<td>25.0</td>
</tr>
</tbody>
</table>
populations during homogenization. For this purpose, factor levels needed are 1 cycle, 2 atm of inlet pressure, and an ionic strength of 22 mM. With these settings, the experimental mean diameter is 319 ± 6 nm, with a width of 83.2 ± 13.4 nm (n = 3). In this case, the values predicted by the model are 338 nm in the case of the mean diameter and 397 nm in the case of the width. Observe that in both unimodal suspensions, the measured mean diameters are comparable with the estimated values, when considering the standard error of estimate (Table II) and the experimental variability. This does not occur in the case of width. This fact can be explained by taking into account that the surface responses are obtained mainly from bimodal samples. During homogenization, the experimental mean diameter

Fig. 1. Surface responses of the mean diameter and width of the size distribution of liposomes obtained with the Microfluidizer 110S. In the case of (A) and (B), the ionic strength is 88 mM. In the case of (C) and (D), the inlet pressure is 2.4 atm.
(a measure of central tendency) will always decrease as a consequence of vesicle size diminution and, consequently, the model will predict the mean diameter of both the unimodal and bimodal samples. On the other hand, the width (which depends on the dispersion of the samples) can show diminutions and increments during the vesicle downsizing if unimodal and bimodal samples are obtained during the process. In the case of samples processed for 1 cycle at 2 atm and at an ionic strength of 22 mM, the corresponding region of the width surface has been obtained from bimodal samples, and, consequently, the predicted value is considerably different from the experimental value (a local minimum is not included in the equation). In the case of samples processed for 9 cycles at 4 atm and at an ionic strength of 22 mM, the difference between the experimental and the estimated width is not as high as in the previous case. This could be caused by the fact that, in this region, the samples used to calculate the width surface are already mainly unimodal.

Liposome Homogenization in Recirculation Mode

Principle

When the Microfluidizer operates in the recirculating mode, the processed parts of the suspension are mixed with the sample contained in the reservoir. Therefore, the system is similar to a continuous stirrer tank reactor with the same inlet and outlet flow rates, where mixing takes place between liposomes that have passed a different number of times through the interaction chamber. With a sufficient period of time, all liposomes experience a disruption and, if the sample is extensively homogenized, vesicles reach the minimum diameter value allowed by the pressure of processing and by sample characteristics. For a constant flow rate (in the case of homogenizers depending on the pressure), the time evolution of this type of system depends on the volume of the sample being processed.

Method

Liposome suspensions are obtained by pouring and mixing sodium phosphate buffer (10 mM, pH 7.4) with Pro-Lipo-S. The phospholipid concentration of all samples is 50 mg/ml and they are processed at 4 atm of inlet pressure at room temperature. The spool valve is selected in recirculating mode and the sample volumes processed are 15, 30, 45, 60, and 90 ml (in all cases $n = 3$). As the maximum volume of the sample reservoir is 25 ml, a 400-ml sample reservoir is installed when needed. In these cases, in order to produce optimal mixing in the reservoir, mechanical stirring
is applied internally by means of flat plates. At specific time intervals, aliquots of 0.2 ml are taken from the sample reservoir and are analyzed with the UPA 150. Maximum homogenizing times range from 4 min, in the case of 15-ml samples, to 10 min in the case of 90-ml samples.

**Results**

During homogenization, the mean diameter diminishes in all samples over time until reaching a constant value. This value, $28 \pm 7$ nm ($n = 15$), is significantly different ($p < 0.05$) from that obtained through processing the same type of sample for 9 cycles at 4 atm ($39 \pm 7$ nm; see previous results) but shows neither a practical nor statistically significant difference if their corresponding widths of size distribution are taken into account ($13 \pm 4$ and $15 \pm 4$ nm, respectively). Therefore, the constant mean diameter reached in all cases corresponds to the minimum vesicle diameter that can be obtained at the operating pressure. All results have a good fit to a time exponential decay (Table III), with the next general expression being

$$\text{Mean diameter (nm)} = d_m + A \exp(-t/\tau) \quad (7)$$

where $t$ is time in seconds, $d_m$ equals 28 nm (this is not allowed to vary during the fitting procedure and, consequently, becomes the horizontal asymptote), and $A$ (nm) and $\tau$ (s) are the parameters specified by the fitting procedure.

Parameter $\tau$ is equivalent to the residence time used in describing time evolution in continuous stirrer tank reactors and, as shown in Fig. 2, has a good correlation with the sample volume. Because of this fact, and considering that the curve passes through the coordinate origin, all equations

<table>
<thead>
<tr>
<th>Sample volume (ml)</th>
<th>$A$ (nm)</th>
<th>$\tau$ (s)</th>
<th>$\sigma$ (nm)</th>
<th>$\sigma$ (s)</th>
<th>$R^2$</th>
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<tbody>
<tr>
<td>15</td>
<td>1087</td>
<td>16.81</td>
<td>0.26</td>
<td>24</td>
<td>0.9714</td>
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<tr>
<td>30</td>
<td>1062</td>
<td>33.2</td>
<td>3.91</td>
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<td>0.9075</td>
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<tr>
<td>45</td>
<td>1099</td>
<td>59.5</td>
<td>3.80</td>
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<td>0.9626</td>
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<td>60</td>
<td>1079</td>
<td>81.9</td>
<td>4.35</td>
<td>18</td>
<td>0.9832</td>
</tr>
<tr>
<td>90</td>
<td>1051</td>
<td>104</td>
<td>8.6</td>
<td>26</td>
<td>0.9282</td>
</tr>
</tbody>
</table>

*Fitted parameters of the exponential decay of the mean diameter when liposomes are processed with recirculation in the Microfluidizer 110S (value standard deviation; $n = 3$).
describing mean diameter variation [Eq. (7)] can be expressed as a function of time/volume, becoming

\[
\text{Mean diameter (nm)} = d_m + A \exp\left(-\frac{t}{1.242V}\right)
\]

(8)

where \( V \) is the sample volume in milliliters.

Experimental results using Eq. (8) are shown in Fig. 3A. The absolute time scale is obtained by multiplying the \( x \) axis values by the volume of the processed sample.

The width distribution shows similar behavior to the mean diameter (Fig. 3B). For any time greater than a critical value, the width diminishes until it becomes constant (13 4 nm; \( n = 15 \)). This width is not significantly different (\( p > 0.05 \)) from the minimum width obtained through processing the sample for 9 cycles at 4 atm and at an ionic strength of 22 mM (15 4 nm; see previous results). As in the case of the mean diameter, all experiments show a comparable evolution when represented as a function of \( t/V \) (Fig. 3B). At the outset of homogenization, all samples show an increment of the width because of the mixing of small quantities of processed sample with the suspension contained in the sample reservoir. Consequently, the width increment reflects an initial increment of the vesicle size distribution. After a maximum width value of approximately 0.7 s/ml is reached (Fig. 3B, inset), the suspension decreases in width, becoming mainly unimodal when small liposomes are obtained.
Effect of Temperature on Liposome Homogenization

**Principle**

The thermotropic properties of phospholipids determine membrane characteristics such as, for example, permeability\(^{17}\) and stability.\(^{19,20}\) Because of membrane rigidity below the phase transition temperature


some methods of liposome preparation must be performed at a temperature higher than the bilayer $T_c$, otherwise devices employed become blocked because, normally, the sample cannot circulate. Although in the case of high-pressure homogenizers this condition need not be accomplished when working at moderate phospholipid concentrations and high pressures, temperature is an important factor affecting vesicle size, depending on the membrane $T_c$.

Method

Liposome suspensions ($n = 4$) with unsaturated phospholipids at a concentration of 10 mg/ml are obtained by pouring and mixing Emulmetik 930 with water at 55°C for 1 h. Liposomes made from saturated phospholipids ($n = 3$) at the previous concentration are obtained from Emulmetik 950, pouring and mixing with water for 1 h at 60°C. All samples are homogenized for 1 cycle at 4 atm of inlet pressure.

The processing temperature is controlled by means of three procedures: first, the Microfluidizer coil is immersed in a water bath at the selected temperature. Second, before processing the sample, water at the chosen temperature is recirculated in the Microfluidizer in order to preheat the circuit. After this, and in order to avoid sample dilution, the water contained in the reservoir and within the pipes is taken out (e.g., by suction with a plastic pipette). Third, the sample is heated to the required temperature and placed in the reservoir.

The processed samples are analyzed with the UPA 150. As reference, three replicates of 18 ml of each type of liposome suspension are processed extensively in the recirculation mode at 23°C and 4 atm of inlet pressure for 10 min.

The phase transition temperature of suspensions obtained by stirring is studied by differential scanning calorimetry (DSC) with an MC2 microcalorimeter (Microcal, Northampton, MA). Before being placed in the cell, sample aliquots are diluted with water at a final phospholipid concentration of 4 mg/ml. The scan rate is 90°C/h and temperature ranges from 20 to 80°C.

Results

DSC measurements of liposomes obtained from saturated phospholipids ($n = 3$) by stirring show a pretransition peak centered at 47.0 ± 0.4°C, a main transition temperature at 52.9 ± 0.1°C, and a small shoulder at 58.6 ± 0.3°C. The last transition may reflect size inhomogeneity in the vesicle population, as confirmed by size analysis with the UPA 150 (size

distribution is bigger than 1000 nm and a part is higher than 6.5 \( \mu \text{m} \), the maximum range of device analysis). Liposomes from unsaturated phospholipids show no phase transitions \( (n = 3) \).

It should be pointed out that homogenization causes a pressure-dependent increase in the temperature of the sample processed. In the case of 1 cycle of homogenization and between 0 and 4 atm of inlet pressure, the temperature increment is 2.12\(^\circ\)/atm times the inlet pressure \( (r^2 = 0.9933) \). Thus, considering that phospholipid-specific heat during phase transitions has no effect on suspension-specific heat temperature because of low sample concentration, and that water-specific heat is practically constant between 23 and 73\(^\circ\) (changing from 4.1804 to 4.1972 J \( \text{g}^{-1} \text{\degree C}^{-1} \), respectively), the temperature of the samples immediately after 1 cycle of homogenization at 4 atm is about 8\(^\circ\) higher than that of the bath.

The effect of the (bath) temperature on the mean diameter of liposomes made from unsaturated phospholipids is shown in Fig. 4. When samples are processed for 1 cycle at 4 atm, there is no variation of mean diameter (or of width) on temperature increase. In the case of samples obtained by recirculation at 4 atm and at room temperature (Fig. 4, open circle), liposome mean diameter is significantly lower than that of the previous samples. These phenomena indicate that the constant liposome size achieved at 4 atm and 1 cycle in the studied temperature range is caused only by the

![Fig. 4. Variation of liposome mean diameter with homogenization temperature. Circles: Samples obtained from unsaturated soybean phospholipids. Squares: Samples obtained from soybean hydrogenated phospholipids with a main transition temperature of 52.9\(^\circ\). Solid symbols: Samples processed for 1 cycle at 4 atm of inlet pressure at the indicated temperature. Open symbols: Samples of 18 ml processed for 10 min in the recirculation mode at room temperature at 4 atm of inlet pressure.](image-url)
fact that the temperature has no effect on the vesicles, because, for example, at this pressure and at room temperature liposomes can be efficiently downsized by the homogenizer if the sample is recirculated.

Unlike previous suspensions, the size distribution of liposomes from saturated phospholipids obtained by homogenization for 1 cycle at 4 atm shows temperature dependence. From room temperature to approximately 45°C, liposome mean diameter is close to 1000 nm. If the temperature increases, size distribution splits into two populations: one initially centered at the previous diameter, and another at approximately 65 nm. The higher the temperature the bigger the population of small-diameter liposomes. Concomitantly, a decrease in the size of the large-diameter liposomes is observed. These facts cause a progressive diminution of the measured mean diameter. After this vesicle size decrement inside the phase transition temperature range, the size distribution of liposomes becomes constant for any temperature higher than about 60°C. In these cases, the small-diameter population of liposomes is approximately 33%. Results reflect the effect of the bilayer phase state on the size reduction capability of the Microfluidizer. As reference, samples processed in the recirculation mode for 10 min at 4 atm at 23°C (open square) show that, at this inlet pressure, liposome size can be controlled by homogenization at a temperature below the $T_c$. Under these conditions, a monomodal distribution of liposomes is obtained.

**Effect of Ethanol and Phospholipid Concentration on Liposome Homogenization**

**Principle**

As a consequence of the method of preparation or because biological implications are being studied, some liposome suspensions contain ethanol in the bulk medium. Some standard procedures for the production of liposomes, such as injection methods, the Pro-Lipo-S system, or the preparation of giant vesicles, involve the presence of ethanol in different steps of the processes and its concentration affects the properties of the vesicles obtained. Phospholipid concentration has also shown its influence on the diameter of those liposomes obtained by the ethanol injection method and by high-pressure homogenization. Likewise, liposomes are employed as membrane models to investigate the effect of ethanol concentration on

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the phospholipid bilayer: membrane interdigitation, 24,25 phospholipid dehydration, 26 and ethanol association with membranes 27 have been reported.

Method

Liposome suspensions at a phospholipid concentration of 160 mg/g (this unit is used instead of mg/ml, because water–ethanol solutions are not ideal) are obtained from Emulmetik 930 by pouring and mixing with water for 1 h at 55°C. After cooling the suspensions to room temperature, the necessary amounts of ethanol are poured into sample aliquots in order to obtain the desired concentration of alcohol and phospholipid. In the case of ethanol, concentration ranges from 0 to 160 mg/g, and in the case of phospholipids, it ranges from 0 to 40 mg/g. In all cases, the chosen relative concentration of the components allows liposome formation. 28 After processing the aliquots with the Microfluidizer for 2 cycles at 2.4 atm of inlet pressure, the obtained suspensions are analyzed with the UPA 150.

Results

Equations (9) and (10), shown in Table IV, are obtained by fitting the experimental mean diameter and width. Both equations have a significant factor correlation. The results show that, in the studied range, the only significant factor determining particle size is ethanol concentration.

\[
\begin{align*}
\text{Eq. (9)} & \quad \text{Mean diameter (nm)} = 288 - 118E^* \quad R^2 = 0.982 \quad F_{\text{calc}}(F_{\text{crit}} = 4.21) = 86 \quad \text{Standard error of estimate} = 102 \\
\text{Eq. (10)} & \quad \text{Width (nm)} = 276 - 147E^* \quad R^2 = 0.983 \quad F_{\text{calc}} = 52 \quad \text{Standard error of estimate} = 164
\end{align*}
\]

\( \text{Table IV} \)

**Effect of Ethanol on Liposome Size Distribution**

<table>
<thead>
<tr>
<th>Equation no.</th>
<th>Response</th>
<th>(R^2)</th>
<th>(F_{\text{calc}}(F_{\text{crit}} = 4.21))</th>
<th>Standard error of estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eq. (9)</td>
<td>Mean diameter (nm) = 288 – 118E*</td>
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<td>52</td>
<td>164</td>
</tr>
</tbody>
</table>

*Fitted empirical equations of mean diameter and width and their statistical liposome suspensions results obtained at different ethanol concentrations. The coded value of the ethanol concentration \((E)\) is \(E^* = (E – 80)/40.\)

Therefore, under the fixed operating conditions of the homogenizer, the liposome diameter diminishes with the concentration of the bulk medium ethanol and, at the same time, the vesicle size distribution becomes narrower.

Preparation of Proteoliposomes Containing Bacteriorhodopsin by High-Pressure Homogenization

Principle

Bacteriorhodopsin is a membrane protein with light-driven proton pump activity found in the purple membrane of *Halobacterium salinarum*. Bacteriorhodopsin is the simplest proton pump that, on *in vivo* absorption of light, cause a pH decrease of the outside cell medium. There are certain methods for reconstituting this protein in lipid vesicles (proteoliposomes). As bacteriorhodopsin, either as detergent-solubilized protein or as purple membrane sheets, exhibits spontaneous incorporation into large preformed vesicles, the simplest procedure for obtaining proteoliposomes involves mixing of protein and liposome suspensions. There are other techniques to prepare proteoliposomes, such as sonication, French press, or reconstitution processes that involve the use of detergents. The light-driven net flux of proton transport across the membranes of the proteoliposomes is modulated by several factors including bulk medium pH, and the composition and size of the vesicles, because they affect the preferred orientation of bacteriorhodopsin in the bilayers. This net flux of protons can be specified by measuring the pH changes in the bulk medium.

Method

A dry film of egg yolk phosphatidylcholine (150 mg) is obtained in a round-bottom flask by rotatory evaporation. Twenty-five milliliters of a purple membrane water dispersion with a bacteriorhodopsin concentration of 0.12 mg/ml in 0.15 M KCl, pH 7, is added to the film and vortexed for 10 min in order to obtain large multilayered proteoliposomes (MLPs) caused by mechanical dispersion. A 5-ml aliquot is separated. After eliminating residual water inside the circuit of the Microfluidizer and cleaning it

with a small volume of the sample, the MLP suspension is processed at 23°C for 1 cycle at 4 atm of inlet pressure. An aliquot of this suspension is taken. Finally, 15 ml of the previous suspension obtained by homogenization is now processed in the recirculation mode at 4 atm for 6 min at 23°C, in order to achieve the minimum vesicle diameter.

Before pH measurements, all proteoliposome suspensions are kept in darkness for at least 30 min. This procedure allows to eliminate any pH gradient between the internal and external medium of the vesicles caused by ambient light. All pH measurements are undertaken in dim red light. A 2.5-ml volume of the proteoliposome suspension is transferred to a stirred 3-ml cuvette placed in a thermostatted bath at 23°C. A glass electrode (Crison 52–08) connected to a pH meter (713 pH meter; Metrohm, Herisau, Switzerland) is placed into the suspension. Before illuminating the sample, about 3 min is allowed to elapse in order to permit pH stabilization. Illumination is provided by a light generator (250 PRN; Scem, Barcelona, Spain) equipped with an incandescent halogen lamp of 250 W. The light produced is focused on the cuvette by means of a fiber optic cable, and a yellow cutoff filter is placed between the cuvette and the light output. The resulting light intensity on the sample is about $80 \times 10^3$ lx. Two light/darkness cycles of 3 and 7 min, respectively, are carried out on all samples.

**Results**

The vesicle size distributions of the proteoliposome suspensions are shown in Fig. 5. In the case of MLPs, part of the vesicle population has a diameter beyond the upper range of the UPA 150 analysis (6.5 μm), resulting in a mean diameter of 2059 $\pm$ 329 nm ($n = 5$) and a width of 810 $\pm$ 355 nm. Samples processed for 1 cycle at 4 atm have a highly spread size distribution that includes vesicles with a diameter on the order of 60 nm to vesicles of more than 1 μm. For these suspensions, the mean diameter is 491 $\pm$ 76 nm ($n = 4$) and the width is 540 $\pm$ 64 nm. Recirculated samples have the smallest size distribution, with 71.4 $\pm$ 3 nm for mean diameter ($n = 5$) and 41.6 $\pm$ 2.4 nm for mean width. Figure 6 shows the pH increments for the different proteoliposome suspensions obtained. As the two light/darkness cycles applied to the samples show no significant differences, results are calculated from their means. The number of independent preparations is the same as in the case of vesicle size analyses. On illumination, all the proteoliposome suspensions produce an alkalinization of the external bulk medium, indicating a preferred (but not necessarily a unique) protein orientation in the bilayers, causing a net proton translocation from the external to the internal vesicle space. After illumination, a
Fig. 5. Size distribution of various suspensions of bacteriorhodopsin proteoliposomes obtained by mechanical dispersion ($\triangle$), by processing the previous suspension for 1 cycle at 4 atm with the Microfluidizer 110S ($\square$), or by 6 min of recirculation in the homogenizer at 4 atm ($\bigcirc$).

Fig. 6. Time evolution of the bulk medium pH of different proteoliposome suspensions under changing illumination conditions. The vesicle size distribution is the same as that indicated in Fig. 5. In all cases, the EPC concentration is 6 mg/ml, and the EPC-to-bacteriorhodopsin ratio is 50:1 (w/w). Results are expressed as the mean (symbols) and as positive or negative standard deviation (error bars).
pH decay is observed in all cases, being caused by passive proton diffusion from the internal aqueous space of the vesicles to the external bulk medium. MLP suspensions show a low pH increase, reaching the maximum pH value in approximately 20 s. No pH changes are detected on illumination for any time greater than 20 s. It is worth mentioning that, with this type of vesicle, when experiments are carried out that illuminate the cuvette by using a second fiberoptic cable placed opposite the first, the same results are obtained. Consequently, the low pH changes observed with MLP suspensions are not caused by the high light scattering of the samples that could act as a filter, decreasing the light across the cuvette. Compared with the previous vesicles, all homogenized proteoliposomes show a high pH increment, which is maximum in the case of recirculated vesicles. In both cases, the stationary state is not reached during the 3-min illumination period.

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[5] Liposome Preparation by Detergent Removal

By Rolf Schubert

Introduction

Detergents can be defined as the particular subgroup of surfactants that are able to solubilize lipid membranes. Sufficient amounts of detergents lead to the reorganization of lipid bilayers to form smaller, soluble detergent–lipid aggregates of various shapes, which are called mixed micelles (MMs). The reverse way, that is, when the amount of detergents in MMs is reduced, leads to a successive enlargement of the MMs. At a critical detergent-to-lipid ratio membrane bilayers are formed, which spontaneously vesiculate to form liposomes. At distinct intermediate phases of detergent–membrane lipid aggregation, membrane proteins can be reconstituted into the membrane bilayers.