

# Vesicle–micelle structural transition of phosphatidylcholine bilayers and Triton X-100

Alfonso DE LA MAZA\* and Jose Luis PARRA

Departamento de Tensioactivos, Centro de Investigación y Desarrollo (C.I.D.), Consejo Superior de Investigaciones Científicas (C.S.I.C.), C/. Jorge Girona, 18–26, 08034 Barcelona, Spain

The structural transition stages induced by the interaction of the non-ionic surfactant Triton X-100 on phosphatidylcholine unilamellar vesicles were studied by means of static and dynamic light-scattering, transmission-electron-microscopy (t.e.m.) and permeability changes. A linear correlation was observed between the effective surfactant/lipid molar ratios ( $Re$ ) ('three-stage' model proposed for the vesicle solubilization) and the surfactant concentration throughout the process. However, this correlation was not noted for the partition coefficients of the surfactant between the bilayer and the aqueous medium ( $K$ ). Thus a sharp initial  $K$  increase was observed until a maximum value was achieved for permeability alterations of 50% (initial step of bilayer saturation). Further surfactant additions resulted in a fall in the  $K$  values until 100% of bilayer permeability. Additional amounts of surfactant led to an increase in  $K$  until bilayer solubilization. Hence, a preferential incorporation of surfactant molecules into liposomes governs the initial interaction steps, leading to the initial stage of bilayer saturation with a free surfactant concentration that was lower than its critical micelle concentration (c.m.c.). Additional amounts of surfactant increased the free surfactant until the c.m.c. was reached, after which solubilization started to occur. Thus the initial step of

bilayer saturation was achieved for a smaller surfactant concentration than that for the  $Re_{sat}$ , although this concentration was the minimum needed for solubilization to start. Large unilamellar vesicles began to form as the surfactant exceeded 15 mol% (50% bilayer permeability), the maximum vesicle growth being attained for 22 mol% (400 nm). Thereafter, static light-scattering started to decrease gradually, this fall being more pronounced after 40 mol%. The t.e.m. picture for 40 mol% ( $Re_{sat}$ ) showed unilamellar vesicles, although with traces of smaller structures. From 50 mol% the size distribution curves began to show a bimodal distribution. The t.e.m. pictures for 50–64 mol% revealed tubular structures, together with open bilayer fragments. Thereafter, increasing amounts of surfactant (65–69 mol%) led to planar multilayered structures which gradually tended to form concentric and helicoidal conformations. The scattered intensity decreased to a low constant value at more than 71–72 mol%. However, the surfactant concentration for the  $Re_{sol}$  (72.6 mol%) still presented traces of aggregated structures, albeit with monomodal size-distribution curves (particle size of 50 nm). This vesicle size corresponded to the liposome solubilization via mixed-micelle formation.

## INTRODUCTION

Many studies have been devoted to the understanding of the principles governing the interaction of surfactants with phospholipid bilayers which leads to the breakdown of lamellar structures and the formation of lipid–surfactant mixed micelles [1–7]. A significant contribution has been made by Lichtenberg [8], who postulated that the critical effective surfactant/lipid ratio ( $Re$ ) producing saturation and solubilization depends on the surfactant critical micellar concentration (c.m.c.) and on the bilayer/aqueous medium distribution coefficients ( $K$ ) rather than on the nature of the surfactants. Moreover, Lichtenberg [8] expressed the need for experimental data on the distribution coefficients at sub-solubilizing surfactant concentrations to obtain complementary information on this complex phenomenon.

The solubilization and reconstitution of lipid bilayers by surfactants have been the subject of a number of mechanistic speculations where open bilayer fragments appear as crucial intermediates both in the closure [9–11] and solubilization processes [6,12]. The mechanisms on this transition are far from understood, since a detailed description of the process has yet to be given. The non-ionic surfactant Triton X-100 has, because of its properties as a good solubilization agent for membrane proteins, been the subject of a number of studies [13–16]. In

earlier papers we studied some parameters implicated in the interaction of surfactants with liposomes at subsolubilizing and solubilizing concentrations [17–19]. In the present work we seek to extend our investigations by characterizing in detail the overall process involved in the interaction of a Triton X-100 with phosphatidylcholine (PC) neutral liposomes, at both sub-solubilizing and solubilizing levels. To this end, we present t.e.m. pictures and vesicle-distribution curves of Triton X-100/phosphatidylcholine systems for different stages of this interaction. This information, together with the comparative study of the  $Re$  and  $K$  parameters throughout the process, may enhance our understanding of the complex phenomenon involved in the lamellar-to-micelle transition process of liposome solubilization and reconstitution by surfactants.

## EXPERIMENTAL

### Materials

PC was purified from egg lecithin (Merck, Darmstadt, Germany) by the method of Singleton [20] and was shown to be pure by t.l.c. The non-ionic surfactant Triton X-100 ( $T_{X-100}$ ), octylphenol polyethoxylated with 10 units of ethylene oxide and active matter of 100%, was purchased from Rohm and Haas (Lyon, France). Pipes buffer obtained from Merck was prepared as 10 mM Pipes

Abbreviations used: t.e.m., transmission electron microscopy; c.m.c., critical micelle concentration; PC, phosphatidylcholine;  $T_{X-100}$ , Triton X-100; CF, 5(6)-carboxyfluorescein; f.i.d., flame-ionization detection; P.I., polydispersity index.

\* To whom correspondence should be sent.

adjusted to pH 7.20 with NaOH, containing 100 mM Na<sub>2</sub>SO<sub>4</sub>. Polycarbonate membranes and membrane holders were purchased from Nucleopore (Pleasanton, CA, U.S.A.). The starting material 5(6)-carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY, U.S.A.) and further purified by a column chromatographic method [21].

## Methods

### Liposome preparation

Unilamellar liposomes of a defined size (about 200 nm) were prepared by extrusion of large unilamellar vesicles previously obtained by reverse-phase evaporation [22,23]. A lipidic film was formed by removing the organic solvent by rotatory evaporation from a solution of PC in chloroform. The lipid was then redissolved in diethyl ether, and Pipes buffer was added to the solution of phospholipid (supplemented with 10 mM CF dye when studying bilayer permeability). Gentle sonication led to the formation of a W/O-type emulsion. After evaporation of the diethyl ether under reduced pressure a viscous gel was formed. Elimination of the final traces of the organic solvent at high vacuum transformed the gel into a liposome suspension in which no traces of ether were detectable by n.m.r. [24]. Unilamellar vesicles were obtained by extrusion of vesicle suspensions through 800–200 nm polycarbonate membranes to achieve a uniform size distribution [25]. To study the bilayer permeability changes, vesicles containing CF were freed of unencapsulated fluorescent dye by passage through Sephadex G-50 medium resin (Pharmacia, Uppsala, Sweden) by column chromatography. The range of phospholipid concentration in liposomes was 0.5–5.0 mM.

### Determination of lipid bilayer concentration and particle size distribution

The phospholipid concentration (*PL*) of liposomes was determined using t.l.c. coupled to an automated flame-ionization-detection (f.i.d.) system (Iatroscan MK-5; Iatron Lab., Tokyo, Japan) [26].

The vesicle size distribution and the polydispersity index (P.I.) of liposomes after preparation and during the interaction with T<sub>x-100</sub> was determined with a Photon correlator spectrometer (Malvern Autosizer 4700c PS/MV). The studies were made by particle-number measurement at 25 °C and with a lecture angle of 90°. After preparation, vesicle-size distribution varied very little (PC concentration from 0.5–5.0 mM), showing in all cases a similar value of about 200 nm (P.I.) lower than 0.1, thereby indicating that the size distribution was very homogeneous.

### Solubilizing parameters

When defining the parameters related to the solubilization of liposomes it is essential to consider that the mixing of lipids and surfactants is not ideal due to the specific interactions between both components, which has been demonstrated for a variety of amphiphiles [27,28]. In order to evaluate the alterations caused by the T<sub>x-100</sub> on lipid bilayers, the effective surfactant/PL molar ratio *Re* in an aggregate (liposome or micelle) is defined as follows [8]:

$$Re = \frac{[\text{total surfactant}] - [\text{surfactant monomer}]}{[\text{total PL}] - [\text{PL monomer}]} \quad (1)$$

The second term of the denominator is negligible due to the low solubility of PL in water. Likewise, it is generally admitted that an equilibrium partition of surfactants between bilayer and the

aqueous medium governs the incorporation of surfactants into liposomes, thereby producing saturation and solubilization of these structures.

In the analysis of the equilibrium-partition model proposed by Schurtenberger [29] for bile salt/lecithin systems, Lichtenberg [8] and Almog et al. [5] have shown that, for a mixing of lipids [at a PL concentration *PL* (mM)] and surfactant [at a concentration *S<sub>T</sub>* (mM)], in dilute aqueous media, the distribution of surfactant between lipid bilayers and aqueous media obeys a partition coefficient *K*, given (in mM<sup>-1</sup>) by:

$$K = \frac{S_B}{(PL + S_B) \cdot S_w} \quad (2)$$

where *S<sub>B</sub>* is the concentration of surfactant in the bilayers (mM) and *S<sub>w</sub>* is the surfactant concentration in the aqueous medium (mM). For *PL* ≫ *S<sub>B</sub>*, the definition of *K*, as given by Schurtenberger [29], applies:

$$K = \frac{S_B}{(PL \cdot S_w)} = \frac{Re}{S_w} \quad (3)$$

where *Re* is the above-mentioned ratio of surfactant to phospholipid in the vesicle bilayer: *Re* = *S<sub>B</sub>*/*PL*. Under any other conditions, eqn. (2) has to be employed to define *K*; this yields:

$$K = \frac{RE}{S_w(1 + Re)} \quad (4)$$

This approach is consistent with the experimental data offered by Lichtenberg [8] and Almog [5] for different surfactant phospholipid mixtures over wide ranges of *Re* values. Given that the range of phospholipid concentrations used in our investigation is similar to that used by Almog [5] to test his equilibrium partition model, the *K* parameter has been determined using this equation.

The determination of the *S<sub>w</sub>* and *Re* parameters can be carried out on the basis of the linear dependence existing between the surfactant concentrations required to achieve these parameters and the phospholipid concentration in liposomes which can be described by the equation:

$$S_T = S_w + Re \cdot PL \quad (5)$$

where the *Re* and the aqueous concentration of surfactant (*S<sub>w</sub>*) are in each curve respectively the slope and the ordinate at the origin (zero phospholipid concentration).

The surface tensions of buffered solutions containing increasing concentrations of T<sub>x-100</sub> were measured by the ring method [30] using a Krüss tensiometer. The c.m.c. was determined from the abrupt change in the slope of the surface tension values versus surfactant concentration.

### Permeability alterations and solubilization of liposomes

The permeability alterations caused by T<sub>x-100</sub> were determined by monitoring the increase in the fluorescence intensity of the liposome suspension due to the CF released from the interior of vesicles to the bulk aqueous phase [21]. Fluorescence measurements were made with a Shimadzu RF-540 spectrofluorophotometer. On excitation at 495 nm, a fluorescence maximum emission of CF was obtained at 515.4 nm. The fluorescence intensity measurements were taken at 25 °C. The percentage of CF released was calculated by means of the equation:

$$\% \text{CF release} = \frac{I_t - I_0}{I_\infty - I_0} \cdot 100 \quad (6)$$

where  $I_0$  is the initial fluorescence intensity of CF-loaded liposome suspension in the absence of surfactant, and  $I_t$  is the fluorescence intensity measured 30 min after adding the surfactant solution to a liposome suspension. This interval was chosen as the minimum period of time needed to achieve a constant rate of CF release for the lipid concentration range used (0.5–5.0 mM). The experimental determination of this interval of time is indicated in the Results and discussion section.  $I_\infty$  corresponds to the fluorescence intensity remaining after the destruction of liposomes.

With regard to liposome solubilization, it has been previously demonstrated [31] that static light-scattering constituted a very convenient technique for the quantitative study of the bilayer solubilization by surfactants. Accordingly, the solubilizing perturbation produced by  $T_{X-100}$  in PC liposomes was monitored using this technique. The overall solubilization can be mainly characterized by three parameters termed  $Re_{sat}$ ,  $Re_{50\%}$  and  $Re_{sol}$ , according to the nomenclature adopted by Lichtenberg [32] corresponding to the  $Re$  ratios at which light-scattering starts to decrease, reaches 50% of the original value and shows no further decrease. These parameters corresponded to the  $T_{X-100}$ /lipid molar ratios at which the surfactant: (a) saturated liposomes, (b) resulted in a 50% solubilization of bilayers and (c) led to a complete solubilization of these structures.

Liposomes were adjusted to the adequate lipid concentration (from 1.0 to 10.0 mM). Equal volumes of the adequate surfactant solutions were added to these liposomes and the resulting mixtures were left to equilibrate for 24 h. This time was chosen as the optimum period needed to achieve a complete equilibrium surfactant/liposome for the lipid concentration range used [31].

Light-scattering measurements were made using the spectrofluorophotometer at 25 °C with both monochromators adjusted to 500 nm. The assays were carried out in triplicate and the results given are the average of those obtained.

#### Electron microscopy

A Hitachi H-600AB transmission electron microscope operating at 75 kV was used. Carbon-coated copper/palladium grids G-400 mesh, 0.5 Taab with 0.5% E 950 collodium films in n-pentyl acetate were employed. A drop of the vesicular solution was sucked off the grid and, after 1 min, removed with filter paper down to a thin film. Negative staining with a drop of a 1% solution of uranyl acetate was performed. After 1 min this drop was again removed with filter paper and the resulting stained film was dried in a dust-free place.

## RESULTS AND DISCUSSION

### Parameters involved in the $T_{X-100}$ -liposomes interaction

It is known that, in surfactant/lipid systems, complete equilibrium may take several hours [8,31]. However, in subsolubilizing interactions a substantial part of the surfactant effect takes place within approx. 30 min after its addition to the liposomes [14].

In order to determine the time needed to obtain a constant rate of CF release of liposomes in the range of the phospholipid concentration investigated (0.5–5.0 mM), a kinetic study of the interaction of liposomes with Triton X-100 was carried out. Liposome suspensions were treated with different concentrations of Triton X-100 at subsolubilizing concentrations and subsequent changes in permeability were studied as a function of time. The permeability kinetics were similar for each system tested: about 30 min was needed to achieve a constant rate of CF release. Hence, changes in permeability were studied 30 min after addition

of surfactant to the liposomes at 25 °C. The CF release of liposome suspensions in the absence of surfactant 30 min after preparation was negligible.

In order to determine the partition coefficients of surfactant between aqueous media and the lipid bilayers, a systematic investigation of liposome permeability changes caused by the addition of surfactant was carried out. To this end, changes in the CF released from liposomes versus surfactant concentration were determined 30 min after surfactant addition at 25 °C. The results obtained are plotted in Figure 1. The surfactant concentrations resulting in different percentages of CF release were obtained graphically and plotted versus the phospholipid concentration. An acceptable linear relationship was established in each case. The straight lines obtained correspond to the aforementioned eqn. (5) from which the  $Re$  and  $K$  parameters were determined. These results including the free surfactant concentration  $S_w$  and the regression coefficients of the straight lines are given in Table 1. An increasing tendency of  $Re$  was observed as the percentage of CF release increased. However,  $K$  reached a maximum value for the CF release of about 50%. Furthermore, the  $S_w$  values increased as the percentage of CF released rose. Bearing in mind that the  $T_{X-100}$  c.m.c. experimentally obtained was 0.15 mM, the  $S_w$  values were in all cases lower than its c.m.c., thereby confirming that permeability alterations were determined by the action of surfactant monomer.

In accordance with the procedure described by Urbaneja [31], the  $T_{X-100}$ -liposomes solubilizing interaction was studied through the changes in the static light scattered by these systems 24 hours after the addition of surfactant. Figure 2 shows the solubilization curves of liposome suspensions (lipid concentration 0.5–5.0 mM) arising from the addition of increasing amounts of  $T_{X-100}$ . At low surfactant concentration an initial increase in the static light-scattering was observed, in all cases due to the incorporation of surfactant molecules into bilayers, the maximum values being reached for bilayer saturation. Increasing amounts of surfactant led to a fall in the scattered intensity until a low constant value for bilayer solubilization was attained. The surfactant concentrations producing different light-scattering percentages were obtained by graphical methods. Plotting the surfactant concentration versus the lipid concentration, curves were obtained in which an acceptable linear relationship was also established in each case. The corresponding  $Re$  and  $K$  parameters were determined from these straight lines (eqn. 5) and are also given in

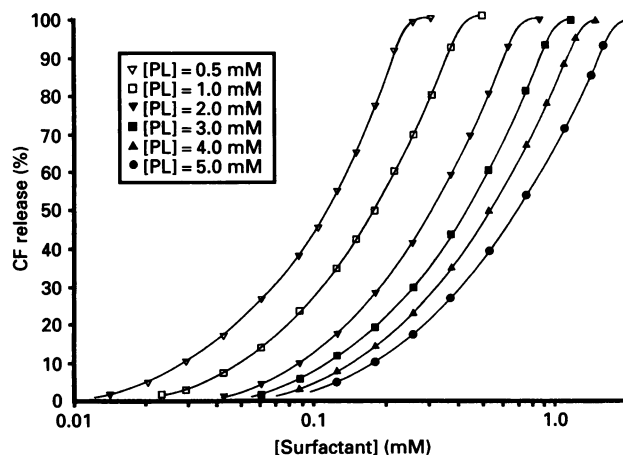


Figure 1 Percentage change in CF release induced by  $T_{X-100}$  on unilamellar PC liposomes, the bilayer lipid concentration ranging from 0.5 to 5.0 mM

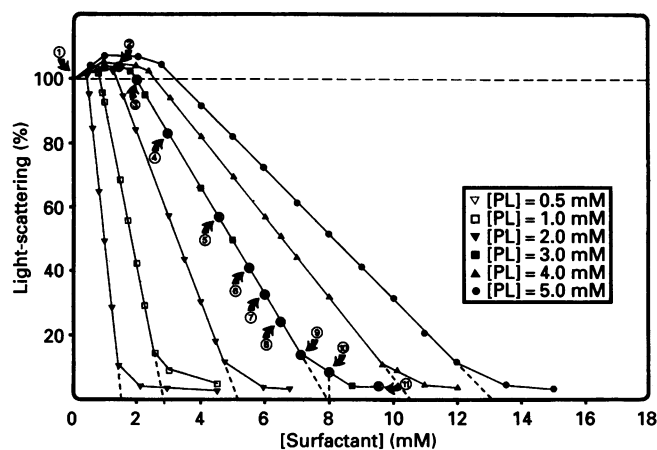
**Table 1** Surfactant to phospholipid molar ratios ( $Re$ ), partition coefficient ( $K$ ) and surfactant concentration in the aqueous medium ( $S_w$ ) resulting in the overall interaction of  $T_{x-100}$  with PC liposomes

The regression coefficients  $r^2$  of the straight lines obtained are also included.

(a)	CF release (%)	$S_w$ (mM)	$Re$ (mol/mol)	$r^2$	$K$ ( $\text{mM}^{-1}$ )
	10	0.015	0.029	0.988	1.883
	20	0.020	0.052	0.990	2.476
	30	0.025	0.077	0.990	2.862
	40	0.033	0.11	0.995	3.005
	50	0.041	0.150	0.997	3.184
	60	0.050	0.188	0.993	3.164
	70	0.055	0.210	0.992	3.157
	80	0.065	0.254	0.997	3.116
	90	0.070	0.273	0.989	3.063
	100	0.090	0.353	0.987	2.900

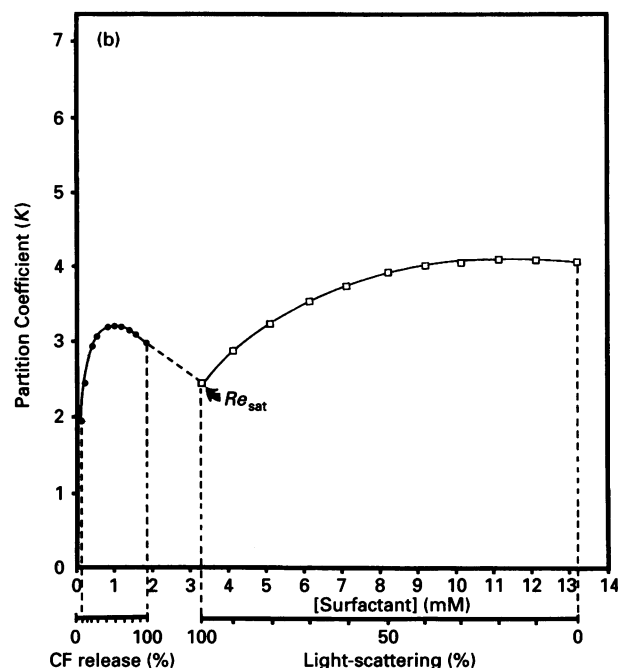
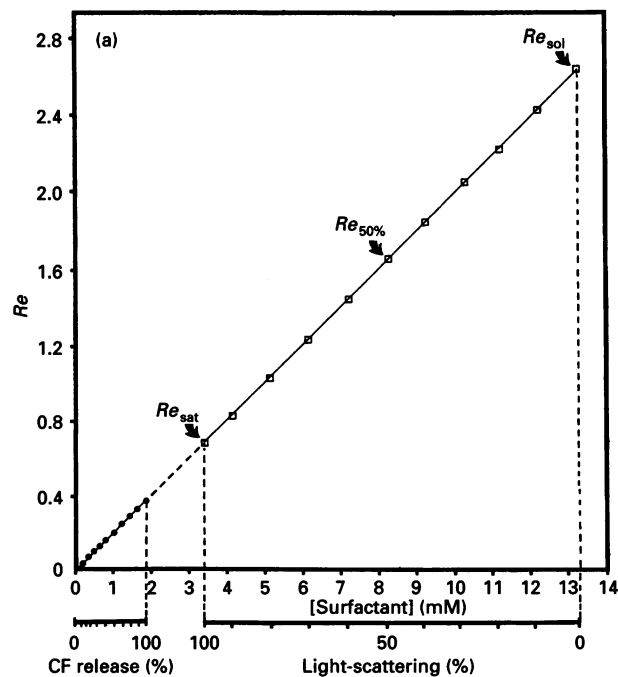
  

(b)	Light-scattering (%)	$S_w$ (mM)	$Re$ (mol/mol)	$r^2$	$K$ ( $\text{mM}^{-1}$ )
	100	0.16	0.64	0.990	2.439
	90	0.16	0.80	0.990	2.777
	80	0.16	1.0	0.991	3.125
	70	0.16	1.20	0.992	3.409
	60	0.16	1.40	0.994	3.645
	50	0.16	1.62	0.993	3.864
	40	0.165	1.80	0.999	3.896
	30	0.17	2.00	0.993	3.921
	20	0.17	2.20	0.989	4.044
	10	0.175	2.40	0.988	4.033
	0	0.18	2.60	0.989	4.012



**Figure 2** Percentage change in static light-scattering of PC liposomes, the lipid concentration ranging between 0.5 and 5.0 mM, versus  $T_{x-100}$  surfactant concentrations

Table 1. It should be noted that, in solubilizing processes, the  $Re$  values increased as the static light-scattering percentage decreased, the values obtained for  $Re_{sat}$  and  $Re_{sol}$  being in agreement with those reported by Paternostre et al. [22]. A similar tendency was observed for the  $K$  values, despite showing a maximum in the final solubilizing stages (from 20 to 0% of light-scattering).



**Figure 3** Plots of (a) Variation in the effective surfactant to phospholipid molar ratio ( $Re$ ) versus surfactant concentration during the overall interaction of  $T_{x-100}$ /PC liposomes, the lipid bilayer concentration remaining constant (5.0 mM) and (b) variation in partition coefficient ( $K$ ) versus surfactant concentrations during the overall interaction of  $T_{x-100}$ /PC liposomes, the bilayer lipid concentration also remaining constant (5.0 mM)

This parameter during solubilization may be regarded as a dynamic equilibrium between the different transition structural stages from lipid bilayers to mixed micelles. Furthermore, the free surfactant concentration ( $S_w$ ) was always similar to the  $T_{x-100}$  c.m.c.

Figure 3 shows the variation in  $Re$  (Figure 3a) and the  $K$  (Figure 3b) parameters versus the surfactant concentration,

**Table 2 Vesicle size distributions (nm) and polydispersity indexes of  $T_{X-100}$ /PC systems when increasing the surfactant concentration, the lipid concentration remaining constant (3.0 mM)**

The ten samples are numbered in accordance with the  $T_{X-100}$ /PC systems (numbered samples) indicated in Figure 2.

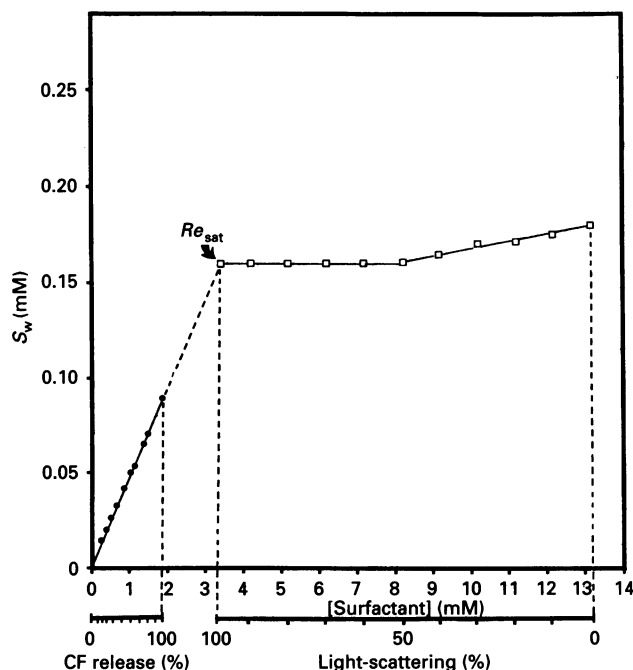
Sample	Type	Curve distribution (vesicle number)				Average Mean (nm)	P.I.
		First peak		Second peak			
		(nm)	(%)	(nm)	(%)		
1	M*	—	—	200	23.2	200	0.112
2	M	—	—	400	23.1	400	0.180
3	M	—	—	355	23.3	355	0.210
4	B†	50	3.8	345	19.8	298	0.250
5	B	50	5.2	328	18.3	266	0.249
6	B	50	7.0	258	16.8	197	0.231
7	B	50	9.0	190	14.8	137	0.215
8	B	50	9.3	119	14.6	92	0.205
9	B	50	19.1	105	4.4	60	0.180
10	M	50	23.6	—	—	50	0.170

\*M, monomodal.

†B, bimodal.

throughout the  $T_{X-100}$ -liposomes interaction, the lipid concentration remaining constant (5.0 mM). The surfactant concentrations producing subsolubilizing and solubilizing alterations are also indicated. It is noteworthy that a linear correlation was obtained between the  $Re$  values and the surfactant concentration throughout the interaction, i.e., the higher the surfactant concentration in the system, the greater the concentration in the bilayers. As for the  $K$  parameter (Figure 3b) a marked initial increase was observed as the  $T_{X-100}$  concentration rose, reaching a maximum for CF release value of about 50%. Increasing surfactant concentrations resulted in a progressive fall in the  $K$  values until 100% of CF release. The extrapolation of the curve (discontinuous line) led approx. to the initial  $K$  value for solubilization (100% light-scattering). Successive additions of surfactant resulted again in a rise in  $K$ , despite showing almost a constant value in the vicinity of the total solubilization of liposomes.

From the results of Figure 3a, it may be assumed that the ratio of  $T_{X-100}$  to phospholipid in the vesicle bilayers or in the mixed micelles appears to be independent of any possible structural organization of both components throughout the process. Comparison of Figures 3a and 3b shows that a significant variation in the free surfactant concentration  $S_w$  took place during the different stages of the interaction with respect to the amount of surfactant molecules incorporated in bilayers. Thus the marked initial increase in  $K$  may be correlated with the larger tendency of surfactant molecules to be incorporated into bilayers with respect to the aqueous phase, this tendency reaching the highest point for approx. 50% of CF release (maximum  $K$  value). The evolution of these two parameters in the subsequent interaction steps (from 50 to 100% of CF release) reveals that a significant variation in the free surfactant concentration ( $S_w$ ) took place with respect to the amount of surfactant molecules incorporated in the bilayers. Thus a gradual increase in  $S_w$  occurs, due to the establishment of a new equilibrium in the surfactant partition between bilayers and the aqueous medium. The surfactant/lipid ratio corre-

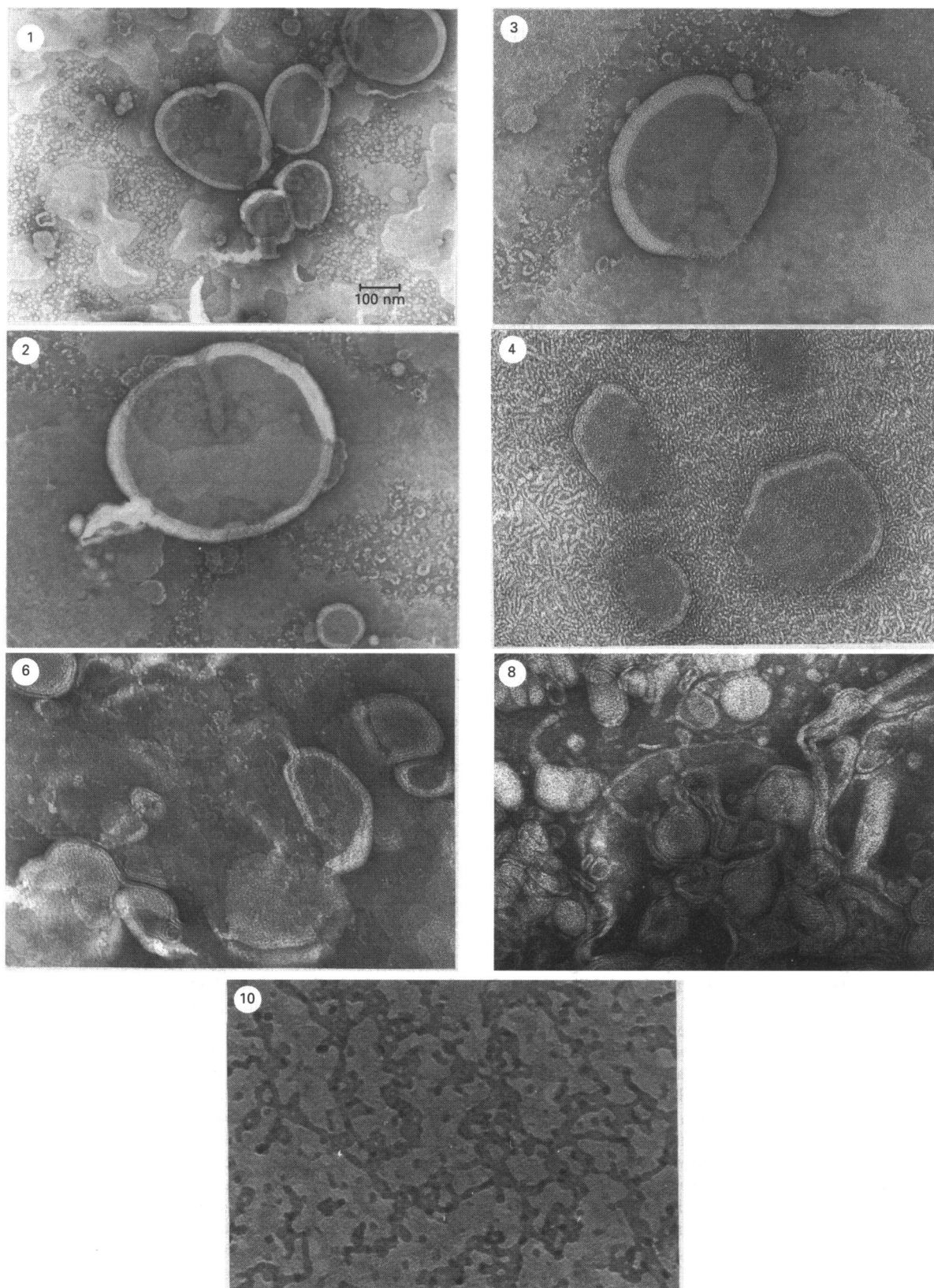


**Figure 4 Plot of the variation in the free surfactant concentration in the aqueous medium ( $S_w$ ) versus the surfactant concentration in the system during the overall interaction of  $T_{X-100}$ /PC liposome suspensions, the lipid concentration remaining constant (5.0 mM)**

sponding to 50% of CF release may be regarded as an important transitional stage which is correlated with both the initial step of bilayer saturation and the establishment of that new partition equilibrium prior to bilayer solubilization. Furthermore, the linear increase of  $Re$  in that interval also reveals that a linear incorporation of surfactant in bilayers occurs until complete saturation of these structures is reached. Both the bilayer saturation and the increase in  $S_w$  occur simultaneously and are reached in the same transitional steps. Surfactant amounts greater than that for 100% of light-scattering again resulted in a gradual increase in  $K$ , i.e. the main bulk of surfactant molecules was again incorporated into bilayers to form mixed micelles until total bilayer solubilization, the aqueous surfactant remaining almost constant with a concentration similar to that of the c.m.c.

The variation in  $S_w$  versus the surfactant concentration for the same lipid concentration (5.0 mM) is plotted in Figure 4. It may be seen that, in subsolubilizing stages, the  $S_w$  increased linearly with surfactant concentration. Comparison of Figures 3b and 4 shows that the initial step of bilayer saturation (50% of CF release) was attained for  $S_w$  values of 0.041 mM, which was clearly lower than the surfactant c.m.c. (0.15 mM). The extrapolation of the  $S_w$  values when increasing the surfactant concentration led to the initial  $S_w$  value for bilayer solubilization (100% light-scattering), which was similar to the surfactant c.m.c. As discussed above, further additions of surfactant resulted in a very low increase in  $S_w$ . The slight inflexion observed in the curve is not significant because of the extremely small  $S_w$  values.

Thus a preferential incorporation of surfactant molecules into liposomes governs the initial stages of this interaction, leading to the initial step of bilayer saturation for a surfactant concentration lower than its c.m.c. Additional amounts of surfactant increases the free surfactant until it reaches similar values to the sur-



**Figure 5** T.e.m. photographs of  $T_{x-100}/PC$  systems when increasing the concentration of the surfactant, the lipid concentration remaining constant (3.0 mM)

The seven pictures shown are numbered in accordance with the  $T_{x-100}/PC$  systems (numbered samples) indicated in Figure 2.

factant c.m.c. Solubilization starts to occur at this moment. Thus the  $Re_{sat}$  parameter given in Figures 3 and 4 corresponds to the exact surfactant/phospholipid molar ratio needed for the initiation of the bilayer solubilization via mixed-micelle formation. The surfactant concentration corresponding to this  $Re_{sat}$  for each lipid bilayer concentration may be considered the c.m.c. of the new surfactant/phospholipid mixed system formed.

In our opinion, the tendency observed in the extrapolated curves of Figures 3 and 4 could shed some light on the linearity existing in both the achievement of the complete bilayer saturation and the rise in the  $S_w$  values until the surfactant c.m.c. Probably a certain compromise between the bilayer saturation and the formation of pure surfactant mixed micelles could be envisaged.

#### Dynamic light-scattering determinations and t.e.m. pictures

A systematic investigation based on dynamic light-scattering measurements and t.e.m. observations was carried out in order to elucidate the structural transition stages involved in the  $T_{x-100}$ -liposomes interaction. Eleven representative surfactant/liposome systems were studied. The corresponding experimental points are indicated in the curve of the static light scattered by 3.0 mM PC liposomes in Figure 2. The vesicle size distribution (nm) and the P.I. of each system is given in Table 2. Figure 5 shows some representative t.e.m. microphotographs of these systems.

For pure vesicle preparations (point 1) the t.e.m. picture showed unilamellar bilayers, the vesicle size-distribution curves giving a monomodal distribution (200 nm). Large unilamellar vesicles began to form as the surfactant concentration exceeded 15 mol %, which corresponds to the 50–60 % of CF release. (The  $T_{x-100}$  mol % used to indicate concentration was based on the total amount of PC and Triton in the sample). The maximum growth of vesicles (correlated with maximum static light-scattering intensity of Figure 2) was observed for 22 mol % (point 2), which roughly corresponds to the 90 % of CF release (unilamellar vesicle size of about 400 nm; P.I. 0.180), the curve showing a monomodal distribution.

It is interesting to note that large unilamellar vesicles began to form when the subsolubilizing partition coefficient,  $K$ , attained approx. the maximum value ( $K = 3.184$ ; 50 % CF release). As discussed above, this surfactant/lipid system corresponds to the initial step of bilayer saturation. These observations also confirm the maximum increase in vesicle size for a surfactant concentration that was lower than that for  $Re_{sat}$  and for smaller  $S_w$  than the surfactant c.m.c. Increasing surfactant concentrations showed the presence of vesicles of similar size, despite a slight fall in the size took place for 40 mol % (point 3 corresponding to the  $Re_{sat}$  parameter). Thus the vesicle size reached values of about 350–360 nm (P.I. of about 0.210) and the t.e.m. picture revealed slightly smaller unilamellar vesicles albeit with initial traces of smaller structures.

When the surfactant concentration reached 50 mol % (point 4) a progressive decrease in static light-scattering occurred, the size distribution curves starting to show a bimodal distribution. Thus a sharp distribution curve appeared at approx. 50 nm, which corresponded to a new particle size distribution (P.I. of about 0.250). At this point, the t.e.m. pictures showed tubular structures together with bilayer fragments. These structures were observed until the surfactant reached approx. 60 mol % (point 5). Thereafter the open structures were gradually affected by the surfactant and progressively worn away. However, for 64 mol % (point 6), the t.e.m. picture revealed the presence of a new type of lamellar aggregate, the size distribution also showing bimodal

curves with an increase in the small particle size (7.0 % of 50 nm particles). The  $Re_{50\%}$  parameter appears to be approximately the minimum surfactant concentration needed to obtain this new type of aggregate.

Surfactant concentrations from 65 to 69 mol % (points 7 and 8) led to the formation of multilayered structures which progressively tended to form concentric and helicoidal conformations. The t.e.m. picture for sample 8 showed the maximum level of complexity where no closed structures were formed, despite the formation of discs and helicoidal aggregates. The size distribution curves still indicated an increase in the small particle size (9.0–9.3 % in 50 nm vesicle size; mean 137–92 nm; P.I. 0.215–0.205). When surfactant exceeded 71 mol % [points 9, (71.4 mol %) and 10 (72.6 mol % which corresponds to  $Re_{so1}$ )], the scattered intensity gradually decreased to a constant low value for bilayer solubilization. The size-distribution curves of point 9 still showed bimodal distribution (mean particle size of about 60 nm and P.I. 0.180), whereas point 10 again showed a single distribution (particle size of about 50 nm and P.I. 0.170). The t.e.m. picture of point 10 still showed some aggregated structures with respect to the mixed micelles obtained at higher surfactant concentrations. It is interesting to note that the  $Re_{so1}$  parameter did not correspond exactly with the total solubilization of bilayers.

#### CONCLUSIONS

From our results we conclude that in the overall interaction of  $T_{x-100}$ /PC liposomes the  $Re$  parameter appears to be directly correlated with the surfactant concentration present in the system, regardless of any possible structural organization involved in this interaction (lipid bilayer structures or mixed micelles). This finding emphasizes the importance of the molecular interaction of both components in this process. However, given the non-linear variation in the  $K$  parameter versus the surfactant concentration, we may assume that a significant variation in the free surfactant concentration takes place throughout the process. Thus the initial increase in  $K$  may be correlated with the initial tendency of the surfactant molecules to be incorporated into bilayers with respect to the surrounding aqueous phase until bilayer saturation. The initial step of bilayer saturation was attained when the system achieved 50 % of CF release and also for free surfactant concentrations smaller than the c.m.c. This transition stage may be correlated with the establishment of a new surfactant partition equilibrium, which leads to complete bilayer saturation and to  $S_w$  values similar to the surfactant c.m.c. Solubilization starts to occur at this moment ( $Re_{sat}$ ).

The t.e.m. observations and the vesicle-size-distribution curves show that large unilamellar vesicles began to form when the subsolubilizing partition coefficient,  $K$ , attained the maximum subsolubilizing value ( $K = 3.184$ ; 50 % CF release). The maximum growth of vesicles was achieved for the transitional step of 90 % CF release (22 mol % surfactant concentration) and, consequently, for a lower surfactant concentration than that for the  $Re_{sat}$  parameter. However, a slight fall in the vesicle size occurred when the surfactant concentration reached the  $Re_{sat}$ , the t.e.m. pictures still showing unilamellar vesicles, albeit with traces of smaller structures. The  $Re_{sat}$  parameter corresponds to the exact surfactant/phospholipid molar ratio needed for the initiation of the bilayer solubilization via mixed-micelle formation. The surfactant concentration corresponding to this  $Re_{sat}$  for each lipid bilayer concentration may be considered the c.m.c. of the new surfactant/phospholipid mixed system formed. Increasing surfactant concentration led to a gradual formation of tubular

structures together with bilayer fragments, followed by the formation of open multilamellar structures, until the formation of mixed micelles began. The surfactant concentration for the  $Re_{50\%}$  seems to be correlated with the formation of these complex structures. The t.e.m. pictures for the  $Re_{sol}$  parameter still show some aggregated structures. This observation confirms that this parameter does not correspond exactly with total bilayer solubilization.

The t.e.m. analysis was performed at Barcelona University, and we thank Dr. Jordi Blavia for his skilful work at the microscope. We are also grateful to Mr. G. von Knorring for expert technical assistance. This work was supported by funds from DGICYT (Dirección General de Investigación Científica y Técnica) (Prog. n° PB91-0065), Spain.

## REFERENCES

- 1 Ollivon, M., Eidelman, O., Blumenthal, R. and Walter, A. (1988) *Biochemistry* **27**, 1695–1703
- 2 Keren-Zur, M., Beigel, M. and Loyter, A. (1989) *Biochim. Biophys. Acta* **983**, 253–258
- 3 Miguel, M. G., Eidelman, O., Ollivon, M. and Walter, A. (1989) *Biochemistry* **28**, 8921–8928
- 4 Levy, D., Gulik, A., Seigneuret, M. and Rigaud, J. L. (1990) *Biochemistry* **29**, 9480–9488
- 5 Almog, S., Litman, B. J., Wimley, W., Cohen, J., Wachtel, E. J., Barenholz, Y., Ben-Shaul, A. and Lichtenberg, D. (1990) *Biochemistry* **29**, 4582–4592
- 6 Edwards, K. and Almgren, M. (1991) *J. Colloid Interface Sci.* **147**, 1–21
- 7 Inoue, T., Yamahata, T. and Shimozawa, R. (1992) *J. Colloid Interface Sci.* **149**, 345–358
- 8 Lichtenberg, D. (1985) *Biochim. Biophys. Acta* **821**, 470–478
- 9 Fromherz, P., Röcker, C. and Rüttel (1986) *Faraday Discuss. Chem. Soc.* **81**, 39–46
- 10 Lasiç, D. D. (1987) *J. Theor. Biol.* **124**, 35–41
- 11 Lasiç, D. D. (1988) *Biochem. J.* **256**, 1–11
- 12 Vinson, P. K., Talmon, Y. and Walter, A. (1989) *Biophys. J.* **56**, 669–676
- 13 Sappey, D., Letoublon, R. and Delmau, J. (1988) *J. Lipid Res.* **29**, 1237–1243
- 14 Ruiz, J., Goñy, F. M. and Alonso, A. (1988) *Biochim. Biophys. Acta* **937**, 127–134
- 15 Edwards, K., Almgren, M., Bellare, J. and Brown, W. (1989) *Langmuir* **5**, 473–478
- 16 Kamenka, N., El-Amrani, M., Appell, J. and Lindheimer, M. (1991) *J. Colloid Interface Sci.* **143**, 463–471
- 17 de la Maza, A., Parra, J. L., García, M. T., Ribosa, I. and Sánchez Leal, J. (1992) *J. Colloid Interface Sci.* **148**, 310–316
- 18 de la Maza, A. and Parra, J. L. (1992) *Langmuir* **8**, 2422–2426
- 19 de la Maza, A. and Parra, J. L. (1993) *Langmuir* **9**, 870–873
- 20 Singleton, W. S., Gray, M. S., Brown, M. L. and White, J. L. (1965) *J. Am. Oil Chem. Soc.* **42**, 53–57
- 21 Weinstein, J. N., Ralston, E., Leserman, L. D., Klausner, R. D., Dragsten, P., Henkart, P. and Blumenthal, R. (1986) in *Liposome Technology* (Gregoriadis, G., ed.), vol. 3, pp. 183–204, CRC Press, Boca Raton, FL
- 22 Paternostre, M. T., Roux, M. and Rigaud, J. L. (1988) *Biochemistry* **27**, 2668–2677
- 23 Rigaud, J. L., Paternostre, M. T. and Bluzat, A. (1988) *Biochemistry* **27**, 2677–2688
- 24 Allen, T. M. (1986) in *Liposome Technology* (Gregoriadis, G., ed.), vol. 1, pp. 109–122, CRC Press, Boca Raton, FL
- 25 Mayer, L. D., Hope, M. J. and Cullis, P. R. (1986) *Biochim. Biophys. Acta* **858**, 161–168
- 26 Ackman, R. G., McLeod, C. A. and Banerjee, A. K. (1990) *J. Planar Chrom.* **3**, 450–490
- 27 Tanford, C. (1980) in *The Hydrophobic Effect: Formation of Micelles and Biological Membranes* (Tanford, C., ed.), pp. 14–20, Wiley and Sons, New York
- 28 Hall, D. G. (1987) in *Nonionic Surfactants, Physical Chemistry* (Schick, M. J., ed.) (Surfactant Science Series), vol. 23, pp. 233–296, Marcel Dekker, New York
- 29 Schurtenberger, P., Mazer, N. and Känzig, W. (1985) *J. Phys. Chem.* **89**, 1042–1049
- 30 Lunkenheimer, K. and Wantke, D. (1981) *Colloid Polymer Sci.* **259**, 354–366
- 31 Urbaneja, M. A., Alonso, A., González-Mañas, J. M., Goñy, F. M., Partearroyo, M. A., Tribut, M. and Paredes, S. (1990) *Biochem. J.* **270**, 305–308
- 32 Lichtenberg, D., Robson, R. J. and Dennis, E. A. (1983) *Biochim. Biophys. Acta* **737**, 285–304