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ORIGINAL CONTRIBUTION

A. de la Maza
J.L. Parra

Assembly properties of Triton X-100/phosphatidylcholine aggregates during liposome solubilization

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Dr. A. de la Maza (✉) · J.L. Parra
Departamento de Tensioactivos
C.I.D.-C.S.I.C.
C/Jordi Girona 18-26
08034 Barcelona, Spain

Abstract The assembly properties of the nonionic surfactant Triton X-100 and phosphatidylcholine (PC) aggregates during the overall solubilization process of PC liposome were investigated. Permeability alterations were detected as a change in 5(6)-carboxyfluorescein (CF) released from the interior of vesicles and bilayer solubilization as a decrease in the static light scattered by liposome suspensions. A direct dependence was established between the bilayer/aqueous phase surfactant partition coefficients (K), the growth of vesicles and the leakage of entrapped CF in the initial interaction steps (surfactant to phospholipid molar ratio Re up to 0.2). These changes may be related to the increasing presence of surfactant molecules in the outer monolayer of vesicles. In the Re range 0.2–0.35 the coexistence of a low vesicle growth with a constant increase of CF release may be correlated with the decrease in K (increased rate of flip-flop of surfactant molecules). Furthermore, in the Re range between 0.64 and 2.0 (lytic levels) almost a linear dependence was detected between the composition of these aggregates (Re) and the decrease in both the surfactant-PC aggregate size and the static light scattered by the system. This dependence was not observed in the last solubilization steps (Re range 2.0–2.60) possibly due to the increased formation of mixed micelles in this interval. The fact that the free Triton X-100 concentration at sublytic and lytic levels showed respectively lower and similar values than its critical micelle concentration confirms that permeability alterations and solubilization were determined respectively by the action of surfactant monomer and by the formation of mixed micelles.

Key words Phosphatidylcholine liposomes – Triton X-100 – permeability alterations and bilayer solubilization – carboxyfluorescein release – static light-scattering – surfactant/phospholipid molar ratios – bilayer/aqueous phase surfactant partition coefficient

Abbreviations PC, phosphatidylcholine; PIPES, piperazine-1,4 bis(2-ethanesulphonic acid); $T_{X\text{-}100}$, Triton X-100; CF, 5(6)-carboxyfluorescein; Re, effective surfactant/lipid molar ratio; Re_{SAT}, effective surfactant/lipid molar ratio for bilayer saturation; Re_{SOL}, effective surfactant/lipid molar ratio for bilayer solubilization; S_W, surfactant concentration in the aqueous medium; S_B, surfactant concentration in the bilayers; S_T, total surfactant concentration; K, bilayer/aqueous phase surfactant partition coefficient; K_{SAT}, bilayer/aqueous phase surfactant partition coefficient for bilayer saturation; K_{SOL}, bilayer/aqueous phase surfactant partition coefficient for bilayer solubilization; PL, phospholipid; TLC-FID, thin-layer chromatography/flame ionization detection system; PI, polydispersity index; CMC, critical micellar concentration; r^2, regression coefficient.

Introduction

The nonionic 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 [1–4]. The interaction of this surfactant with simplified membrane models such as phospholipid bilayers leads to the breakdown of lamellar structures and the formation of lipid–surfactant mixed micelles.

A significant contribution in this area has been made by Lichtenberg [5], who postulated that the critical effective surfactant/lipid ratio (*Re*) producing saturation and complete solubilization of these structures depends on the surfactant critical micellar concentration (CMC) and on the bilayer/aqueous medium distribution coefficients (*K*) rather than on the nature of the surfactants.

In earlier papers, we studied the mechanisms governing the solubilization of neutral and electrically charged unilamellar liposomes by a series of octylphenol polyethoxylated surfactants, by Triton X-100 (octylphenol with an average of 10 ethylene oxide units) or by mixtures of this surfactant with sodium dodecyl sulphate [6–8]. In the present work, we seek to extend our investigations by correlating some assembly properties of the Triton X-100/phosphatidylcholine (PC) systems as the leakage of entrapped 5(6)-carboxyfluorescein (CF) in the interior of liposomes, the surfactant-PC aggregate size, the scattered light of the system and the effective molar ratio of surfactant to phospholipid in bilayers (*Re*) during the overall interaction of this surfactant with PC liposomes. This information may enhance our understanding of the selective contribution of the nonionic surfactant Triton X-100 on the supramolecular structures formed during the different interaction steps, which lead to the saturation and the progressive solubilization of these bilayer structures via mixed micelle formation.

Experimental

Materials and methods

The nonionic surfactant Triton X-100 ($T_{X\text{-}100}$) octylphenol polyethoxylated with 10 units of ethylene oxide and active matter of 100% was purchased from Rohm and Haas (Lyon, France). Phosphatidylcholine (PC) was purified from egg lecithin (Merck, Darmstadt, Germany) according to the method of Singleton [9] and was shown to be pure by thin-layer chromatography TLC. Piperazine-1,4 bis(2-ethanesulphonic acid) (PIPES buffer) obtained from Merck was prepared as 10 mM PIPES adjusted to pH 7.20 with NaOH, containing 110 mM Na_2SO_4. Polycarbonate membranes and membrane holders were purchased from Nucleopore (Pleasanton, CA). The starting material 5(6)-carboxyfluorescein, (CF) was obtained from Eastman Kodak (Rochester, NY) and further purified by a column chromatographic method [10].

Unilamellar liposomes of a defined size (about 200 nm) were prepared by extrusion of large unilamellar vesicles previously obtained by reverse phase evaporation [11, 12]. 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 [10]. The range of phospholipid concentration in liposomes was 0.5–5.0 mM, which was determined using thin-layer chromatography (TLC) coupled to an automated ionization detection (FID) system (Iatroscan MK-5, Iatron Lab. Inc. Tokyo, Japan) [13].

The size distribution and the polydispersity index (PI) of liposomes and surfactant-PC aggregates were determined with a photon correlator spectrometer (Malvern Autosizer 4700c PS/MV; Malvern, England). The studies were made by particle number measurement. Samples were adjusted to the appropriate concentration range with PIPES buffer and the measurements were taken at 25 °C and with a lecture angle of 90°.

The surface tensions of buffered solutions containing increasing concentrations of $T_{X\text{-}100}$ were measured by the ring method [14] using the Krüss processor tensiometer K 12 (Krüss GMBH, Hamburg, Germany). The critical micelle concentration (CMC) of the $T_{X\text{-}100}$ was determined from the abrupt change in the slope of the surface tension values versus surfactant concentration showing a value of 0.15 mM.

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 [15, 16]. To evaluate the alterations caused by the $T_{X\text{-}100}$ on phospholipid bilayers, the effective surfactant/lipid molar ratio *Re* in an aggregate (liposome or micelle) is defined as follows [5]:

$$Re = (S_T - S_W)/(PL - PL_{mon}) \quad (1)$$

where *PL* is the lipid concentration (mM), S_T is the total $T_{X\text{-}100}$ concentration (mM) and S_W is the surfactant concentration in the aqueous medium (mM). The monomeric *PL* concentration (PL_{mon}) 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.

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In the analysis of the equilibrium partition model proposed by Schurtenberger [17] for bile salt/lecithin systems, Lichtenberg [18] and Almog et al. [19] have shown that for a mixing of lipids, in dilute aqueous media, the distribution of surfactant between lipid bilayers and aqueous media obeys a partition coefficient K, given in $(mM)^{-1}$ by

$$K = S_B/[(PL + S_B)\cdot S_W] , \quad (2)$$

where S_B is the concentration of surfactant in the bilayers (mM). For $PL \gg S_B$, the definition of K, as given by Schurtenberger, applies:

$$K = S_B/(PL\cdot S_W) = Re/S_W , \quad (3)$$

where Re is the above-mentioned ratio of surfactant to phospholipid in the vesicle bilayer: ($Re = S_B/PL$). Under any other conditions, Eq. (2) has to be employed to define K; this yields:

$$K = Re/[S_W\cdot(1 + Re)] . \quad (4)$$

This approach is consistent with the experimental data offered by Lichtenberg [18] and Almog [19] 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 to test his equilibrium partition model, the K parameter has been determined using this equation.

The determination of Re and S_W 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 S_W are in each curve respectively the slope and the ordinate at the origin (zero phospholipid concentration).

Permeability alterations and solubilization of liposomes

The permeability alterations caused by $T_{X\text{-}100}$ were determined by monitoring the increase in the fluorescence intensity of the liposome suspensions due to the CF released from the interior of vesicles to the bulk aqueous phase [10]. 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 presence of the $T_{X\text{-}100}$ did not affect the direct quenching of the aforementioned spectrofluorophotometric CF signal. 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 systems were left to equilibrate for 30 min. This interval was chosen as the minimum period of time needed to achieve a constant level of CF release. The experimental determination of this interval of time is indicated in the "Results and Discussion Section". The fluorescence intensity measurements were taken at 25 °C. The percentage of CF released was calculated by means of the equation [7]:

$$\%\ \text{CF release} = (I_T - I_0)/(I_\infty - I_0)\cdot 100 , \quad (6)$$

where I_0 is the initial fluorescence intensity of CF-loaded liposome suspension in the absence of $T_{X\text{-}100}$, I_T is the fluorescence intensity measured 30 min after adding the $T_{X\text{-}100}$ to a liposome suspensions and I_∞ corresponds to the fluorescence intensity remaining after the complete destruction of liposomes by the addition of $T_{X\text{-}100}$ (60 μl of 10% vol/vol) aqueous solution to 2.0 ml of liposome suspension [10].

With regard to liposome solubilization, it has been previously demonstrated that static light-scattering constituted a very convenient technique for the quantitative study of the bilayer solubilization by surfactants [20–22]. Accordingly, the solubilizing perturbation produced by $T_{X\text{-}100}$ in PC unilamellar liposomes was monitored using this technique. The overall solubilization can be mainly characterized by two parameters termed Re_{SAT} and Re_{SOL}, according to the nomenclature adopted by Lichtenberg [18] corresponding to the Re ratios at which light-scattering starts to decrease with respect to the original value and shows no further decrease. These parameters corresponded to the $T_{X\text{-}100}$/lipid molar ratios at which the surfactant: a) saturated liposomes and b) 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 [20, 21]. 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.

Results and discussion

Mean vesicle size and stability of liposome suspensions

The mean vesicle size of liposome suspensions after preparation (phospholipid concentration ranging from 0.5 to 5.0 mM) varied little (around 200 nm). The polydispersity index (PI), defined as a measure of the width of the particle

size distribution obtained from the "cumulant analysis" remained in all cases lower than 0.1 indicating that the liposome suspensions showed a homogeneous size distribution in all cases. The size of vesicles after the addition of equal volumes of PIPES buffer and equilibration for 24 h showed in all cases values similar to those obtained after preparation, with a slight increase in the PI (between 0.12 and 0.14). Hence, the liposome preparations appeared to be reasonably stable in the absence of surfactant under the experimental conditions used in solubilization studies.

Interaction $T_{X\text{-}100}$/liposomes

It is known that, in surfactant/lipid systems, complete equilibrium may take several hours [5, 20]. However, in subsolubilizing interactions a substantial part of the surfactant effect takes place within approximately 30 min after its addition to the liposomes [23].

To determine the time needed to obtain a constant level of CF release of liposomes in the range of the phospholipid concentration investigated (0.5 and 5.0 mM), a kinetic study of the interaction of $T_{X\text{-}100}$ with liposomes was carried out. Liposome suspensions were treated with a constant subsolubilizing $T_{X\text{-}100}$ concentration (0.2 mM) and subsequent changes in permeability were studied as a function of time (Fig. 1). It may be seen that about 30 min was needed to achieve a constant level of CF release in all cases despite the fact that approx. the 80% of CF release took place during the initial 10 min. From our previous investigations [7], we may assume that this behavior is possibly due to the release of the fluorescent dye through holes, or channels, created in the membrane and not to bilayer fusion. The incorporation of surfactant monomers to membranes may directly induce the formation of hydrophilic pores in these structures or merely stabilize transient holes, in agreement with the concept of transient channels suggested by Edwards et al. in the surfactant-mediated increase in membrane permeability for different nonionic surfactants [3, 24, 25]. As a consequence, changes in permeability were studied 30 min after addition of $T_{X\text{-}100}$ to the liposome suspensions at 25 °C. The CF release of liposome suspensions in the absence of surfactant in this period of time was negligible.

To determine the surfactant partition coefficients between lipid bilayers and aqueous media at subsolubilizing level, a systematic study of permeability changes caused by the addition of $T_{X\text{-}100}$ to liposomes was carried out for various lipid concentrations. Changes in CF release were determined 30 min after surfactant addition at 25 °C. The results obtained for phospholipid concentration 1.0 mM are plotted in Fig. 2. The surfactant concentrations resulting in different percentages of CF release for each

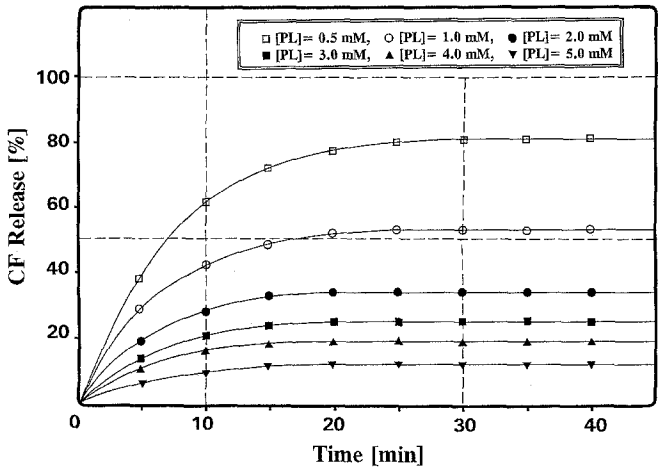

Fig. 1 Time curve of the release of CF trapped in PC liposomes caused by the addition of $T_{X\text{-}100}$ (0.2 mM). The phospholipid concentration of liposomes were $[PL] = 0.5$ mM (□), $[PL] = 1.0$ mM (○), $[PL] = 2.0$ mM (●), $[PL] = 3.0$ mM (■), $[PL] = 4.0$ mM (▲), $[PL] = 5.0$ mM (▼)

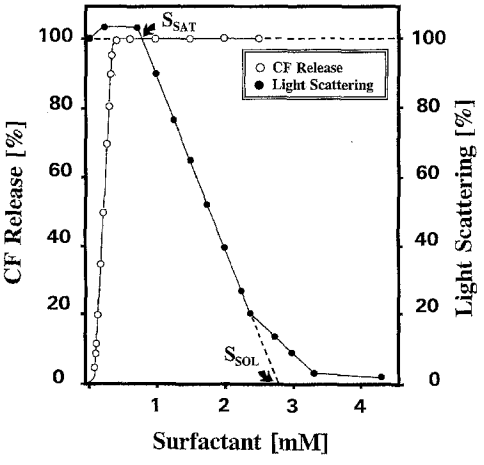

Fig. 2 Percentage changes in CF release (○) and static light-scattering (●) of unilamellar liposomes (lipid concentration 1.0 mM) induced by the presence of increasing concentrations of $T_{X\text{-}100}$. The arrows indicating S_{SAT} and S_{SOL} (static light-scattering curve) corresponded respectively to the surfactant concentration for saturation and solubilization of liposomes

phospholipid concentration tested were graphically obtained and plotted versus the phospholipid concentration (Fig. 3A). The straight lines obtained correspond to the aforementioned Eq. (5) from which Re and S_w were determined. The regression coefficients (r^2) for these straight lines are given in Table 1. This parameter always showed higher values than 0.987, indicating that an acceptable linear relationship was established in each case. The Re_{SAT} and Re_{SOL} values obtained (respectively

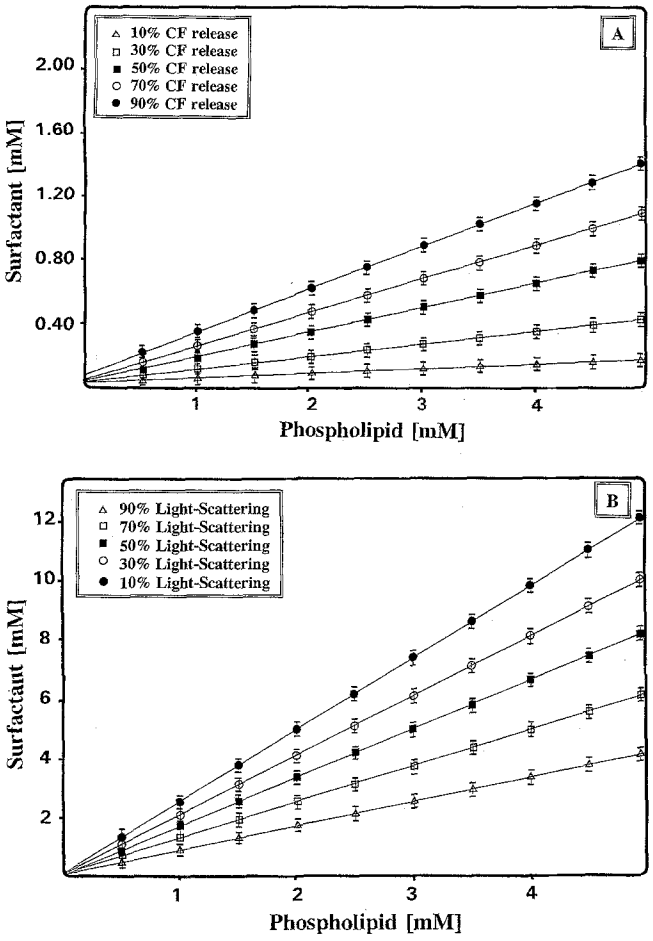

Fig. 3A Surfactant concentrations resulting in different percentages of CF release versus the phospholipid concentration of liposome suspensions. Percentages of CF release; 10% (△), 30% (□), 50% (■), 70% (○), 90% (●); **3B** Surfactant concentrations resulting in different percentages of static light scattering versus the phospholipid concentration of liposome suspensions. Percentages of static light scattering; 90% (△), 70% (□), 50% (■), 30% (○), 10%(●)

Table 1 Regression coefficients of the straight lines corresponding to the different stages of the overall interaction of $T_{X\text{-}100}$ with PC unilamellar liposomes

Regression coefficients r^2			
CF release %	r^2	Light scattering %	r^2
10	0.988	10	0.988
20	0.990	20	0.989
30	0.990	30	0.993
40	0.995	40	0.999
50	0.997	50	0.993
60	0.993	60	0.994
70	0.992	70	0.992
80	0.997	80	0.991
90	0.989	90	0.990
100	0.987	100	0.990

0.64 and 2.60) are in agreement with those previously reported [19, 26].

In accordance with the procedure described by Urbaneja et al., the solubilizing interaction of $T_{X\text{-}100}$ with liposome suspensions was studied through the changes in the static light scattered by these systems 24 h after the addition of surfactant [20, 22]. Figure 2 also shows the solubilization curve of liposome suspensions (PC concentration 1.0 mM) arising from the addition of increasing amounts of $T_{X\text{-}100}$. An initial increase in the scattered intensity of the system was observed due to the surfactant incorporation into bilayers. Additional amounts of surfactant resulted in a fall in this intensity until a low constant value for bilayer solubilization. The arrows indicating S_{SAT} and S_{SOL} corresponded respectively to the surfactant concentration for saturation and solubilization of liposomes. The surfactant concentrations for each phospholipid concentration and corresponding to the different static light scattering percentages were obtained by graphical methods. Plotting this surfactant concentration versus lipid concentration, curves were obtained (Fig. 3B), in which an acceptable linear relationship was also established in each case (r^2 values higher than 0.988, also given in Table 1). The corresponding Re, S_w and K parameters were determined from these straight lines (Eq. (5)).

Relationship between Re and K parameters

Figure 4 shows the variation in K versus Re during the overall surfactant/liposome interaction. A marked initial increase in K was observed as Re rose, reaching a maximum ($K = 3.184$) for $Re = 0.150$ (corresponding to the 50% of CF release). Increasing Re values led to a fall in K values until 100% CF release, this decrease being more pronounced in the interval 60–80% CF release. Thus, the increase in Re resulted in two opposite effects on the surfactant bilayer/water partitioning. At low Re, K first increased, possibly because only the outer vesicle leaflet was available for interaction with surfactant molecules, the binding of additional surfactant to the bilayer being hampered up to approx 80% CF release (abrupt fall in K). The 50% CF release may be correlated with the saturation of the outer vesicle leaflet by the surfactant. Increasing Re (Re between 0.27–0.35 corresponding to a low decrease in K), led to an increased rate of flip-flop of the surfactant molecules, (or permeabilization of the bilayers to surfactant), thus also making the inner monolayer available for interaction with added surfactant. Therefore, the K trend changed due to the presence of surfactant in the bilayers up to saturation of these structures. These findings are in agreement with the results previously reported by Schubert et al. for the interaction sodium cholate with PC

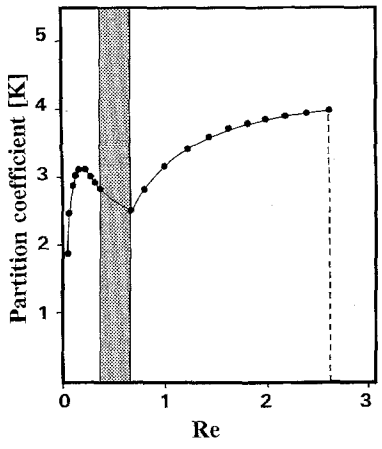

Fig. 4 Variation in the partition coefficients (K) versus the effective surfactant to phospholipid molar ratios (Re) during the overall interaction between the $T_{X\text{-}100}$ and PC liposomes

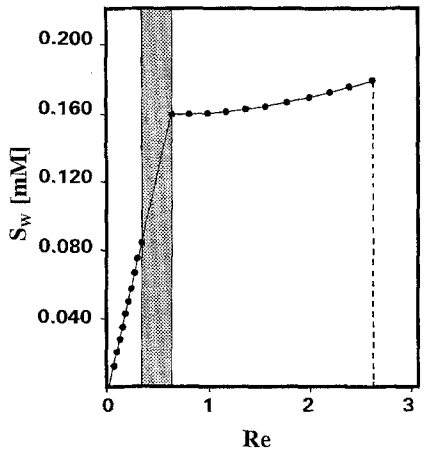

Fig. 5 Variation in the free surfactant concentration (S_w) versus the effective surfactant to phospholipid molar ratio (Re) during the overall interaction between the $T_{X\text{-}100}$ and PC liposomes

liposomes [27]. The extrapolation of the curve (shaded area) led approximately to the initial K value for solubilization (100% light scattering, for Re_{SAT}). From this point the increase in Re resulted again in a rise in K up to Re_{SOL}, which was more pronounced in the Re range 0.64–2.0, and that corresponded to the solubilization of liposomes.

Relationship between the Re parameter and S_w

Figure 5 shows the variation in the surfactant concentration in the aqueous medium (S_w) versus the effective surfactant/lipid molar ratio (Re) throughout the interaction surfactant/liposome (vesicles or mixed micelles). A linear increase in S_w was observed as Re rose up to 100% of CF release ($Re = 0.353$). The extrapolation of the curve (shaded area) led approximately to the initial S_w value for solubilization (100% light scattering, for $Re_{SAT} = 0.64$), which corresponded approx to the surfactant CMC ($S_w = 0.16$ mM and $T_{X\text{-}100}$ CMC 0.15 mM). This finding confirms that permeability alterations were determined by the action of surfactant monomers and supports the generally admitted assumption that the concentration of free surfactant must reach the CMC for solubilization to occur [5,7]. The subsequent increase in Re resulted again in a slight rise in S_w up to Re_{SOL}, which corresponded to the complete solubilization of liposomes via mixed micelle formation.

Dependence of the surfactant-PC aggregate size, CF release and static light scattering on Re

A systematic investigation based on dynamic light-scattering measurements of surfactant-PC aggregates was carried out throughout the process to elucidate the dependencies between the size of these aggregates (vesicles or mixed micelles), the changes in the percentages of both the CF release and the static light scattering of the system and the effective surfactant/lipid molar ratio Re. The values obtained for 5.0 mM PC concentration are given in Table 2. A progressive growth of vesicles was detected as the percentage of CF release rose, the maximum increase was attained for 100% CF release. The growth of vesicles occurred in a few seconds with not much change over a time scale of hours. As for static light scattering variations, the 100% corresponding to the Re_{SAT} produced a fall in the vesicle size albeit with a monomodal distribution. When the light scattered by the system decreased, a sharp distribution curve appeared approx. at 50 nm, which corresponded to a new particle size distribution (PC-surfactant mixed micelles in coexistence with mixed vesicles). The curve for these small particles rose until 10% of scattered light, exhibiting again at this point a monomodal distribution, which corresponded to the surfactant/PC mixed micelles (particles of 50 nm). This increase was especially pronounced in the interval of static light-scattering percentages between 30 and 10% (Re ranging between 1.8 and 2.4).

Figure 6 shows the variation in both the percentage of CF release and the vesicle size of liposome suspensions versus Re at subsolubilizing level. The increase in Re led initially to an almost linear increase in both parameters, these effects being correlated with the increase in K (Fig. 4). As mentioned earlier, this behavior may be correlated with the incorporation of surfactant monomers in the outer bilayer leaflet and its subsequent saturation. However, Re values exceeding 0.2 resulted in a lower growth of vesicles, despite the fact that the maximum

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Table 2 Mean size distributions (nm) and polydispersity indexes of surfactant-PC aggregates (vesicles or mixed micelles) resulting in the overall interaction of $T_{X\text{-}100}$ with PC unilamellar liposomes

CF release %	Curve distribution (particle number)					Average mean (nm)	Polydispersity index
	Type	1st peak		2nd peak			
		nm	%	nm	%		
0	M	–	–	200	100	200	0.112
10	M	–	–	227	100	227	0.123
20	M	–	–	244	100	244	0.128
30	M	–	–	267	100	267	0.135
40	M	–	–	285	100	286	0.142
50	M	–	–	315	100	315	0.149
60	M	–	–	337	100	337	0.158
70	M	–	–	350	100	350	0.162
80	M	–	–	365	100	365	0.170
90	M	–	–	382	100	382	0.175
100	M	–	–	395	100	395	0.182
Light-scattering %							
100	M	–	–	355	100	355	0.210
90	B	50	8.5	331	91.5	307	0.235
80	B	50	15.0	323	85.0	282	0.248
70	B	50	15.3	304	84.7	265	0.234
60	B	50	21.8	293	78.2	240	0.225
50	B	50	32.1	268	67.9	198	0.218
40	B	50	32.8	236	67.2	175	0.212
30	B	50	48.3	222	51.7	139	0.209
20	B	50	69.7	182	30.3	90	0.201
10	M	50	100	–	–	50	0.180
0	M	50	100	–	–	50	0.170

M monomodal, B bimodal.

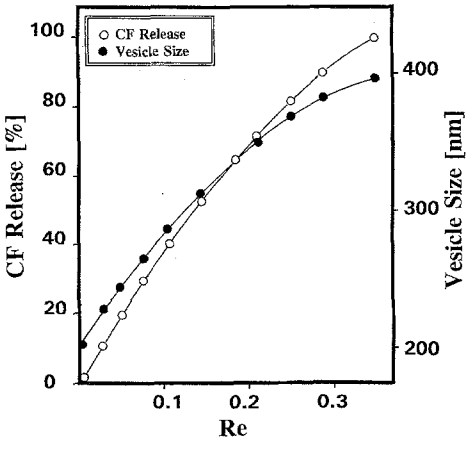

Fig. 6 Variation in the percentage of CF release (○) and vesicle size (●) of liposomes versus *Re* at subsolubilizing level

growth was achieved for 100% of CF (395 nm). Considering that more than 80% of the permeability alterations occurred in the initial interaction step (10 min, Fig. 1) and that the growth of vesicles took place in a few seconds after the addition of surfactant to liposomes, we may assume, in agreement with the results reported by Almog et al. for the nonionic surfactant octyl glucoside [19], that for lower *Re* values than 0.2 the growth of vesicles was dependent on the bilayer composition (*Re*) being also directly correlated with the leakage of entrapped CF. It is interesting to note that in the interval of CF release percentages between 70–100 (*Re* values between 0.2 and 0.35), a similar tendency in the evolution of bilayer permeability occurred although with a low increase in the size of vesicles. These findings may be also correlated with the progressive decrease of *K* in this *Re* interval and consequently, with the aforementioned increased rate of flip-flop of the surfactant molecules (or permeabilization of the bilayers to surfactant).

Figure 7 shows the variation in the percentage of static light scattering and the surfactant-PC aggregate size (average mean) versus *Re* at solubilizing level. The increase in *Re* produced almost a linear decrease in both parameters, i.e., in the range of *Re* values between 0.64 and 2.0 almost a direct correlation between both parameters was established as well as with the composition of the surfactant-PC aggregates. The nonlinear dependence observed for *Re* values higher than 2.0 may be attributed to the fact that in this interval an abrupt increase in the low particle size distribution (50 nm corresponding to the mixed micelle formation) took place (see Table 2). The increased formation of surfactant/PC mixed micelles in the last interaction

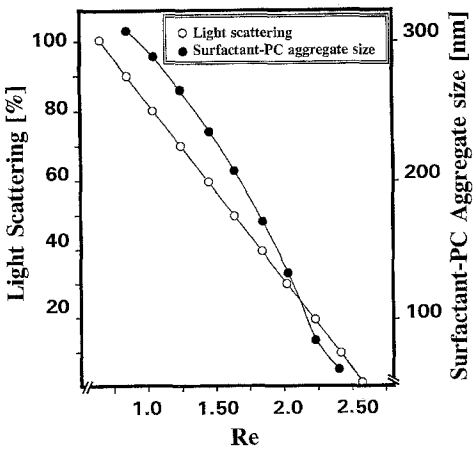

Fig. 7 Variation in the percentage of static light scattering (○) and surfactant-PC aggregate size (●) of surfactant-PC aggregates versus *Re* at solubilizing level

steps contrasts with the progressive formation of these structures during the overall solubilization process and may be considered as a new approach with respect to the results previously reported for this interaction [7].

Comparison of Figs. 7 and 4 shows that in the initial steps of bilayer solubilization (*Re* interval between 0.64 and 2.0) the linear decrease in both the static light scattering and the surfactant-PC aggregate size (average mean) appears to be correlated with the more pronounced increase in K or affinity of surfactant molecules with bilayers. However, the increase in the formation of surfactant/PC mixed micelles in the last solubilization steps (*Re* values between 1.80 and 2.60) is correlated with a low K increase.

Conclusions

From these findings we may conclude that in the initial interaction steps (*Re* up to 0.2) a direct relationship was established between the growth of vesicles, the leakage of entrapped CF and the surfactant bilayer/aqueous phase partition coefficients (K). These changes may be related to the increasing presence of surfactant molecules in the outer monolayer of vesicles. In the *Re* range 0.2–0.35 the coexistence of a low vesicle growth with a constant increase of CF release may be correlated with the decrease in K (increased rate of flip-flop of surfactant molecules). Furthermore, in the *Re* range between 0.64 and 2.0 (lytic levels) almost a linear dependence was detected between the composition of these aggregates (*Re*) and the decrease in both the surfactant-PC aggregate size and the static light scattered by the system. This dependence was not observed in the last solubilization steps (*Re* range 2.0–2.60) possibly due to the increased formation of mixed micelles in this interval. The fact that the free $T_{X\text{-}100}$ concentration at subsolubilizing and solubilizing levels showed respectively lower and similar values than the $T_{X\text{-}100}$ critical micelle concentration (CMC) indicates that permeability alterations and solubilization were determined respectively by the action of surfactant monomer and by the formation of mixed micelles. This finding supports the generally admitted assumption that the concentration of free surfactant must reach the CMC for solubilization to occur.

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