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International Journal of Pharmaceutics 184 (1999) 227–235

international journal of pharmaceutics

Surfactant-induced leakage from liposomes: a comparison among different lecithin vesicles

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Received 23 December 1998; received in revised form 11 March 1999; accepted 12 March 1999

Abstract

The interactions, at sublytic concentration, of Triton X-100 and sodium cholate with sonicated and extruded liposomes of egg and soya lecithins were considered to analyze the integrity and/or the barrier efficiency of liposomal membranes. Results are discussed in terms of surfactant partition between the aqueous and the lipid phases and of the release of a fluorescent hydrophilic probe. Phospholipid nature and liposome size influence detergent partition, whereas the content release is mainly affected by the surfactant mole fraction in the bilayer, and by the liposome size.

Keywords: Surfactant-liposome interactions; Triton X-100; Sodium cholate; Calcein release

1. Introduction

Interactions with surfactants represent one of the most widely investigated topics in the field of liposome research. As already described (Paternostre et al., 1988; Inoue et al., 1992; Lasch, 1995; Keller et al., 1997; Lopez et al., 1998) the phenomena associated with surfactant addition to a liposome dispersion can be described through a 'three-step' model. In the initial stage, when the surfactant concentration is low, surfactant monomers are incorporated within the lipid bilayer according to a partition equilibrium between aqueous and lipid phase; then, when the surfactant concentration exceeds a saturation value, phospholipids are gradually solubilized into mixed micelles that coexist with surfactant-saturated vesicles; finally, complete solubilization of vesicles occurs and only mixed micelles are present in solution. The surfactant concentrations, C^{sat} and C^{sol}, corresponding to the onset and completion of vesicle solubilization, respectively, are frequently utilized to acquire information about the physical stability of the aggregated structure (Ruiz et al., 1988; Ribosa et al., 1992; Elorza et al., 1997).

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0378-5173/99/$ - see front matter
PII: S0378-5173(99)00105-2

Most authors agree with this interpretation of the overall process, but many different interpretations of the detailed mechanism of surfactant-vesicle interactions are reported. In particular, there is no doubt that some leakage of the vesicular content occurs at sublytic detergent concentration, when the vescicle structure is still preserved, but some authors report a slow content release, up to completion (Schubert et al., 1986; Edwards and Almgren, 1990 Lasch et al., 1990; Edwards and Almgren, 1992), while others observe that only a fraction of the entrapped solute is released even after a long time (Ruiz et al., 1988; De la Maza et al., 1992; Goni et al., 1993; De la Maza and Parra, 1994; De la Maza et al., 1998). Differences in behavior have been related to differences in lipid bilayer composition, membrane packing (Nagama and Regen, 1992), liposome size and/or molecular structure of surfactant (Lasch et al., 1990) or surfactant concentration (below or above its critical micelle concentration, Liu and Regen, 1993). Several discrepancies arise from the available data depending on the experimental procedure; therefore the liposome behavior which is actually found and the theoretical analysis from different laboratories can hardly be compared and further investigation seems to be necessary (Inoue, 1996). Moreover it is worth noting that egg phosphatidylcholine is the phospholipid mostly used for these studies whereas much less work has been carried out with other species, although soya phosphatidylcholine is largely used in cosmetic and pharmaceutical industry, also in the presence of surfactants.

In a previous work we compared the physical stability (Memoli et al., 1995) of liposomes prepared according to two different methods with phospolipids of different origin (egg yolk, EPC, and soya bean, SPC) and using Triton X-100 or sodium cholate as solubilizing agents. In the present work we focus our attention on the liposome behavior at sublytic concentration of the same surfactants and we perform a systematic comparison of the content leakage of egg and soya small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV).

2. Materials and methods

2.1. Materials

Ninety percent pure enriched egg phosphatidylcholine (Phospholipon 90 Egg, P90 egg, Natterman Phospholipids GmbH) and 90% pure enriched soya phosphatidylcholine (Phospholipon 90, P90 soya, Natterman Phospholipids GmbH) were used for vesicle preparation.

Hepes pH 7.5 buffer solution (10^{-3} M) prepared with freshly distilled and deareated water was used in all experiments. Sodium cholate, Triton X-100, calcein and all other products used for the present investigation were of analytical grade.

2.2. Methods

Turbidity and fluorescence measurements were carried out using a Perkin Elmer LS5 spectrofluorometer; turbidity measurements were carried out with excitation and emission wavelengths at 600 nm, whereas fluorescence measurements were carried out with excitation and emission wavelengths at 490 and 520 nm, respectively. In the kinetic experiments, data were collected with a 1010 Perkin Elmer Data Station connected with the spectrofluorometer.

Calcein concentration was detected by fluorescence measurements up to 5×10^{-6} M concentration, where calcein self-quenching does not occur. Preliminary experiments showed that Triton X-100 and sodium cholate up to a 0.1% w/v concentration do not affect calcein fluorescence.

2.3. Liposome preparation

SUV and LUV were obtained from large multilamellar vesicles (MLV) prepared by hydration of a dry lipid film (250 mg) with 5 ml of Hepes calcein solution 5×10^{-2} M.

For SUV preparation, the MLV solution was gently shaken for 1 h and then sonicated, under a nitrogen stream, ten times for 5 min (with intervals of 2 min) with a Soniprep 150 apparatus (MSE, Crowley) equipped with an exponential

microprobe. The temperature was maintained constant at 15–20°C by means of a water bath. For LUV preparation, MLV were first gently shaken for 1 h and then freeze-thawed five times with liquid nitrogen and warm water (60°C); the dispersion was finally extruded twice through 200-nm pore size and eight times through 100-nm polycarbonate filters using a Lipex Biomembranes (Vancouver, BC) extruder thermostated at 30°C.

To remove untrapped calcein, the liposome solution was eluted through a Sephadex G75 column, using Hepes buffer. All the vesicles were collected and their presence detected by turbidity measurements and the solution obtained was diluted with HEPES buffer to a fixed lecithin concentration.

Preliminary studies with Phospholipids B test (Wako Chemicals Gmbh) indicated that over 95% of the initial amount of phospholipid used for film preparation was recovered in the form of liposomes.

2.4. Measurements of entrapped volume

Liposome entrapped volume was determined through the measurements of the amount of calcein released, calculated as the difference between the calcein concentration obtained after complete disruption of the vesicles, by Triton X-100, and the calcein concentration in the external solution of intact liposome dispersion. In each preparation, at least five different lecithin concentrations (from 0.001 to 0.04 mg/ml) were checked.

Trapped volumes of all tested preparations are reported in Table 1. It will be noticed that entrapped volumes of the extruded liposomes are larger than those of the sonicated ones, as expected.

2.5. Liposome solubilization

The solubilization process was monitored turbidimetrically as previously reported (Memoli et al., 1995), according to the method described by Paternostre et al. (1988). Different amounts of concentrated surfactant solutions (0.16 M for Triton X-100 and 5×10^{-2} M for sodium cholate) were entered stepwise to 2 ml of liposome suspension at different concentrations, into the stirred fluorimeter cuvette. After each addition, the turbidity was read when the steady state was reached. The two breakpoints observed in the turbidity curves correspond to the onset and completion of the solubilization process, respectively. In the case of sodium cholate the first breakpoint is less sharp (Paternostre et al., 1988), thus it is more difficult to identify a precise C^{sat} value.

2.6. Calcein leakage

Owing to self-quenching, at high intravesicular concentration calcein fluorescence is negligible and leakage through liposome bilayer at different surfactant concentration can be monitored by the fluorescence increase of calcein in the surrounding media.

Different amounts of 0.016 M Triton X-100 or 5×10^{-2} M sodium cholate were added in a stepwise manner to 2 ml of liposome suspension at different concentrations, into the stirred fluorimeter cuvette. After each addition, the fluorescence increase was monitored.

No significant calcein release was detected without detergent addition.

Control experiments carried out by monitoring the fluorescence increase after surfactant addition for 4 h showed that the calcein release is practically completed in the first few minutes.

3. Results and discussion

Turbidity measurements of the solubilization process of all the tested liposome preparations allowed us to evaluate the surfactant concentration required to saturate the bilayer, C^{sat}, and to achieve the complete liposome solubilization, C^{sol}. Both C^{sat} and C^{sol} show a linear dependence on the liposome concentration C_L (expressed as mg of lipids/ml). The regression equations and the corresponding regression coefficients are reported in Table 1, where the lower regression coefficients calculated for Na Cholate C^{sat} should be related to the approximate C^{sat} value, as above reported.

Table 1
Trapped volume and C^{sat} and C^{sol} values for different liposome preparations

Lipids	Liposome preparation	Trapped volume (μl/mg)	Triton X-100				Sodium cholate			
			C^{sat} (mM)	r	C^{sol} (mM)	r	C^{sat} (mM)	r	C^{sol} (mM)	r
Egg	Sonication	0.460 ± 0.022	$0.251 \pm 0.873\ C_L$	0.99	$0.325 \pm 2.81\ C_L$	0.99	$1.285 \pm 2.141\ C_L$	0.85	ND	
Egg	Extrusion	0.906 ± 0.010	$0.164 \pm 0.071\ C_L$	0.80	$0.535 \pm 2.71\ C_L$	0.99	$1.800 \pm 0.437\ C_L$	0.72	15.11	–
Soya	Sonication	0.309 ± 0.011	$0.353 \pm 0.967\ C_L$	0.94	$0.480 \pm 2.81\ C_L$	0.99	$2.507 \pm 1.545\ C_L$	0.75	$11.62 \pm 8.49\ C_L$	0.99
Soya	Extrusion	1.207 ± 0.028	$0.158 \pm 1.220\ C_L$	0.99	$0.287 \pm 2.74\ C_L$	0.99	$0.751 \pm 1.152\ C_L$	0.766	$15.5 \pm 6.29\ C_L$	0.99

According to an already described partition model (Lichtenberger et al., 1983; Lichtenberger, 1985; Schubert et al., 1986), the intercept of the straight line C^{sat} (or C^{sol}) versus C_L provides the free surfactant concentration, C_w, at the onset or completion of the lipid solubilization process while the slope of the same straight line is related to the molar ratio, R, between the surfactant and the lipid in the supramolecular structure (liposomes or mixed micelles, respectively) under saturation or complete solubilization conditions.

Consequently:

$$C^{sat} = C_w^{sat} + (10^{-3}/M_w)R^{sat}C_L$$

or

$$C^{sol} = C_w^{sol} + (10^{-3}/M_w)R^{sol}C_L$$

where M_w is the phospholipid molecular weigh, C_L is expressed as mg of lipids/ml and C_{sat}, C_{sol}, $C_w^{sat}$and C_w^{sol} are expressed as mmol of surfactant/l.

The surfactant partition coefficient, K, defined as the ratio between the surfactant molar fraction in the bilayer and the surfactant concentration in water is therefore given by:

$$K = \frac{R^{sat}}{C_w^{sat}\cdot(1 + R^{sat})}$$

Table 2 reports K and R_{sat} values calculated from Table 1 parameters. As it is evident, EPC extruded preparations, in presence of Triton X-100 shows a partition coefficient and surfactant-to-lipid molar ratio at saturation much lower than those of the other liposomes. An explanation of such behavior could be found taking into account that for the more saturated egg phospholipid composition the liposomes have a less flexible lipid bilayer; therefore, in egg liposomes, surfactant molecule intercalation is more difficult than in the soya ones, which posses a higher unsaturated lipid percentage and, consequently, more flexible bilayers. Furthermore, owing to the low flexibility of the bilayer, a small surfactant-to-lipid molar ratio is sufficient to induce vescicle solubilization. On the other hand, egg sonicated liposomes exhibit higher K and R^{sat} values in agreement with the fact that the incorporation of wedge-shaped surfactant molecules into small unilamellar vescicles (with high bilayer curvature) is easier than in large ones.

As far as liposome saturation and solubilization with sodium cholate is concerned, results reported in Table 2 show a great difference between C_w values corresponding to the bilayer saturation and solubilization. Such a difference, that can be hardly reconciled with the partition model used, has, anyway, been already described for other surfactants (Inoue et al., 1992; Keller et al., 1997). C_w^{sat} values obtained with sodium cholate are largely higher than those obtained with Triton X-100, in agreement with the large difference in CMC values of the two surfactants (14 and 0.24 mM, respectively). Furthermore, the K values obtained for sodium cholate (Table 2) are in the same sequence but always smaller than those obtained with Triton X-100.

Fig. 1 reports the calcein release from the vesicles obtained with different liposome preparations after subsequent additions of Triton X-100, leading to a final surfactant concentration, C, always less than C^{sat}. Results are expressed as the concentration of fluorescent probe in the external solution as a function of time. From the figure it

Table 2
Partition coefficients, K, and surfactant-to-lipid molar ratio at vesicle saturation, R^{sat}, for different liposome preparation

Lipids	Liposome preparation	Triton X-100		Sodium cholate	
		K (mM^{-1})	R^{sat}	K (mM^{-1})	R^{sat}
Egg	Sonication	1.556	0.641	0.476	1.571
Egg	Extrusion	0.302	0.052	0.135	0.321
Soya	Sonication	1.176	0.709	0.212	0.895
Soya	Extrusion	2.990	0.895	0.610	0.845

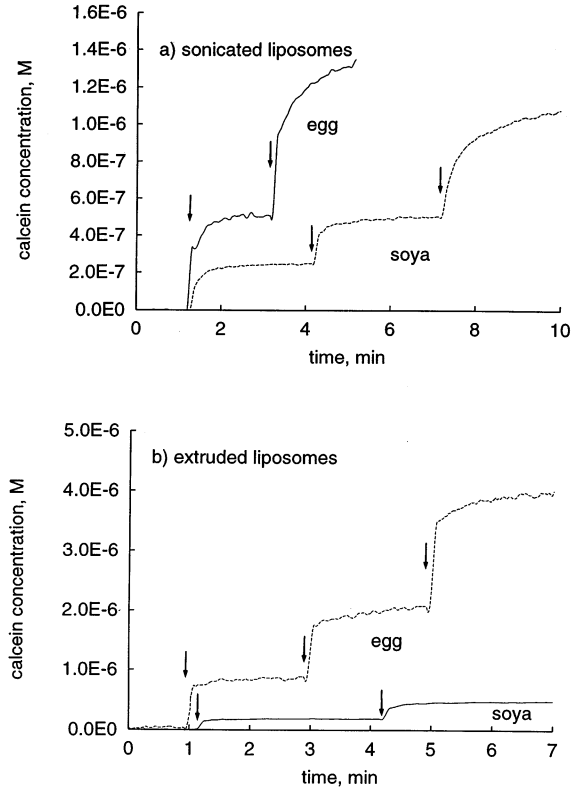

Fig. 1. Kinetics of Calcein release from sonicated (a) and extruded (b) liposomes. Arrows indicate additions of 10 μl of Triton X-100 0.16 M to 2 ml of vesicle dispersion (lipid concentration 0.1125 mg/ml).

is evident that, regardless of the lipid composition or the vescicle size, the trend of calcein release is similar: after the first addition, when the surfactant concentration is far from C^{sat}, calcein release is almost instantaneous and the outer calcein concentration rapidly reaches a stable value for all preparations. After the subsequent additions, when the surfactant concentration approaches the C^{sat} value, a new instantaneous release followed by a reduced but continuous slow leakage is observed, until the complete release is reached. This biphasic trend (fast and slow release) is more evident in the sonicated liposomes. Such behavior seems to suggest that, at low surfactant concentration, detergent molecule insertion into the lipid membrane induces the formation of transient holes; these pores, responsible for the instantaneous release, remain open only for a short time; after that, the barrier efficiency of the membrane is recovered and the calcein efflux stops. When the surfactant concentration becomes higher, a permanent permeabilization of the lipid membrane could occur or the transient holes could stay open long enough to allow a complete release (Edwards and Almgren, 1990). From a quantitative point of view, the amount of calcein released with the same surfactant addition depends on the phospholipid nature and on the vescicle sizes. In the case of SUV (Fig. 1(a)), at the same total surfactant concentration, SPC liposomes show a lower leakage than EPC liposomes; in LUV liposomes (Fig. 1(b)) the opposite behavior is observed. These results agree with the Triton X-100 partition coefficient between the bilayer and the aqueous phase, reported in Table 2: leakage in EPC LUV liposomes is the lowest according with the lower surfactant molecule penetration into the bilayer, but when the curvature radius allows a greater surfactant penetration (EPC SUV liposomes), the leakage is enhanced.

The same behavior is observed in the presence of sodium cholate but, at the same surfactant concentration, the amount of calcein released is always lower than that obtained with Triton X-100. In order to achieve the same release the sodium cholate concentration must exceed the C^{sat} value.

Further information on the surfactant-induced bilayer perturbation can be obtained plotting the percentage of calcein released as a function of each surfactant molar fraction in the bilayer. In fact, the different surfactant partition coefficients (Table 2) result, with the same amount of added surfactant, in different surfactant molar fractions in the bilayer, X_d. Accounting for the surfactant material balance:

$$C = C_w + \frac{X_d}{1 + X_d} C_L$$

where C is the total surfactant concentration and C_w is the free surfactant concentration in water, and the partition coefficient definition ($K = X_d / C_w$), the surfactant molar fraction in the bilayer

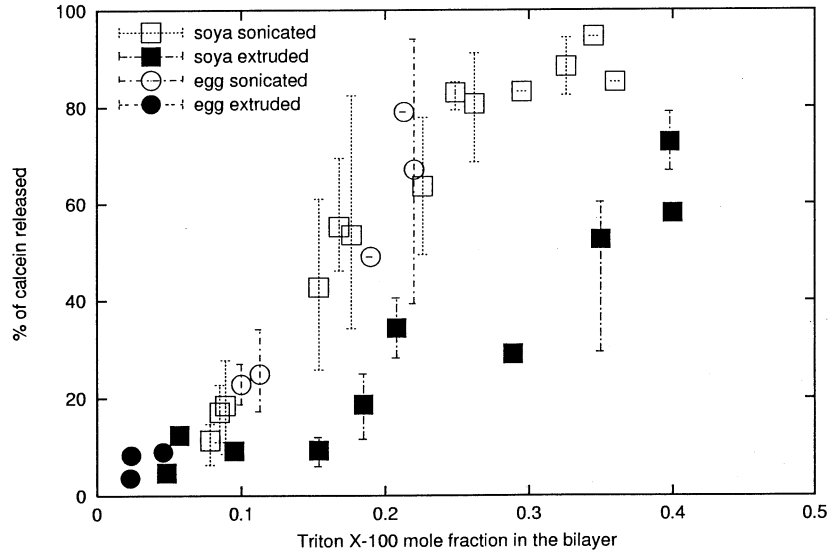

Fig. 2. Percentage of calcein released from egg and soya SUV and LUV as a function of Triton X-100 molar fraction in the bilayer.

is given by:

$$X_d = \frac{(KC + 1 + KC_L) - \sqrt{(KC + 1 + KC_L)^2 + 4KC}}{2}$$

Fig. 2 show the percentage of calcein released in the presence of Triton X-100 for all the lipid preparations tested. The figure suggests, for both sonicated and extruded liposomes, a sigmoidal trend of the release, with a threshold of surfactant mole fraction in the bilayer larger for extruded than for sonicated liposomes (about 0.15 and 0.08 mole fraction, respectively). Furthermore at the same surfactant mole fraction in the bilayer, the percentage of calcein released from soya liposomes obtained by sonication is substantially larger than that obtained from the extruded ones, in agreement with the larger surface-to-volume ratio of sonicated liposomes. In any case significant release is obtained at surfactant mole fraction remarkably lower than that required to saturate and to induce vescicle solubilization and for the sonicated ones also lower than that corresponding to the steep increase of turbidity before saturation.

It is worth noting that, at the same surfactant mole fractions in the bilayer, no significant differences in the percentage of calcein release were observed between soya and egg sonicated liposomes, whereas in the case of egg extruded liposomes, owing to the low C_{sat} value, the release can be determined only in a narrow surfactant concentration range.

In presence of sodium cholate the release is always remarkably lower than that obtained with Triton X-100, even considering the same surfactant molar fraction in the bilayer; in fact, while with Triton X-100 almost complete calcein release is obtained at $C < C_{sat}$, with sodium cholate the percentage of calcein released at sublytic conditions does not exceed 4%.

4. Conclusions

The results obtained from the systematic comparison of the interactions of surfactants with different liposome preparations, can be summarized as follows:

a) the partition coefficients of Triton X-100, ob-

tained from turbidity measurements, show that the ability of this surfactant to intercalate within the lipid bilayer increases as follows:

EPC LUV < SPC SUV < EPC SUV < SPC LUV

Furthermore, in EPC LUV liposomes, the surfactant-to-lipid molar ratio corresponding to the bilayer saturation is about ten times lower than that in the other vesicles. Sodium cholate partition coefficients in different liposomes follow the same sequence order but are always noticeably lower that those of Triton X-100.

b) kinetics of calcein release is qualitatively the same for both surfactants: the release proceeds through a very fast pulsed release, immediately after each surfactant additions at $C << C^{sat}$; when C approaches the C^{sat} value, a subsequent slow release, up to completion, is observed. The first pulsed release is probably due to the transient bilayer rearrangement due to the surfactant addition, whereas the subsequent phase suggests a permanent liposome permeabilization at higher surfactant/lipid ratios.

c) release in the presence of sodium cholate is always much lower than that obtained with Triton X-100, even considering the same surfactant mole fraction in the bilayer.

d) Triton X-100 induced release, at the same surfactant mole fraction in the bilayer, is larger from sonicated vesicles than from extruded ones. No significant difference between egg and soya sonicated vesicles are observed.

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