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Egg and soya phospholipids – sonication and dialysis: A study on liposome characterization

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Abstract

Liposome-Triton X-100 interactions were studied by turbidity measurements and two detergent concentrations, corresponding to the maximum (i.e., detergent-saturated vesicles; R^{sat}) and minimum (i.e., completely disruped liposomes; R^{sol}) turbidity values, were identified. The tested liposomes (SUV) were prepared with phospholipids of different origin (egg and soya) and according to different methods (sonication and dialysis). The data obtained, together with size measurements carried out by electron microscopy, were related to the stability of the vesicles.

Keywords: Small unilamellar vesicles; Egg phospholipid; Soya phospholipid; Sonication; Dialysis

1. Introduction

In previous studies we referred to a number of aspects of the chemical stability (Memoli et al., 1993a) and entrapment capacity (Memoli et al., 1993b, 1994) of liposomes prepared with phospholipids of different origin (egg and soya). Since the physical stability of liposomes is also a problem of great importance (e.g., during employment and storage), we report here some information on the behaviour of small unilamellar vesicles (SUV) prepared with the same materials.

As reported (Ruiz et al., 1988; Ribosa et al., 1992), detergents are commonly used as agents for destroying liposome structures and the information obtained can be directly related to vesicle stability. This interaction with phospholipid liposomes takes place through three main steps (Paternostre et al., 1988), as the surfactant concentration increases. In a first stage, surfactant monomers are incorporated within the lipid bilayer, with a corresponding increase in vesicle dimensions, then phospholipids are gradually solubilized leading to the coexistence of liposomes and mixed micelles and, finally, complete solubilization occurs and only mixed micelles are present. These different stages can readily be followed by turbidity and/or light scattering measurements and two detergent concentrations, corresponding to the maximum (i.e., detergent-saturated vesicles; R^{sat}) and minimum (i.e., com-

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pletely disrupted liposomal structures; R^{sol}) turbidity values, can be identified.

For the present study, one of the most frequently employed surfactants, Triton X-100 (Anzai et al., 1980; Memoli et al., 1993b), was used and the influence of the vesicle composition and the preparation method (sonication and dialysis) on the R^{sat} and R^{sol} values (Sabouni et al., 1992) was investigated.

2. Materials and methods

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

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

For liposomes prepared by sonication, a Soniprep 150 apparatus (MSE, Crowley) equipped with an exponential microprobe was employed, while in the case of the dialysis method a Liposomat flow-through dialyzer (Dianorm) and Diachema (Mol. Wt cut-off 10 000) membranes were used.

Liposome dimensions were evaluated by means of a Philips CM12 transmission electron microscope at 80 kV; liposomes were negatively stained

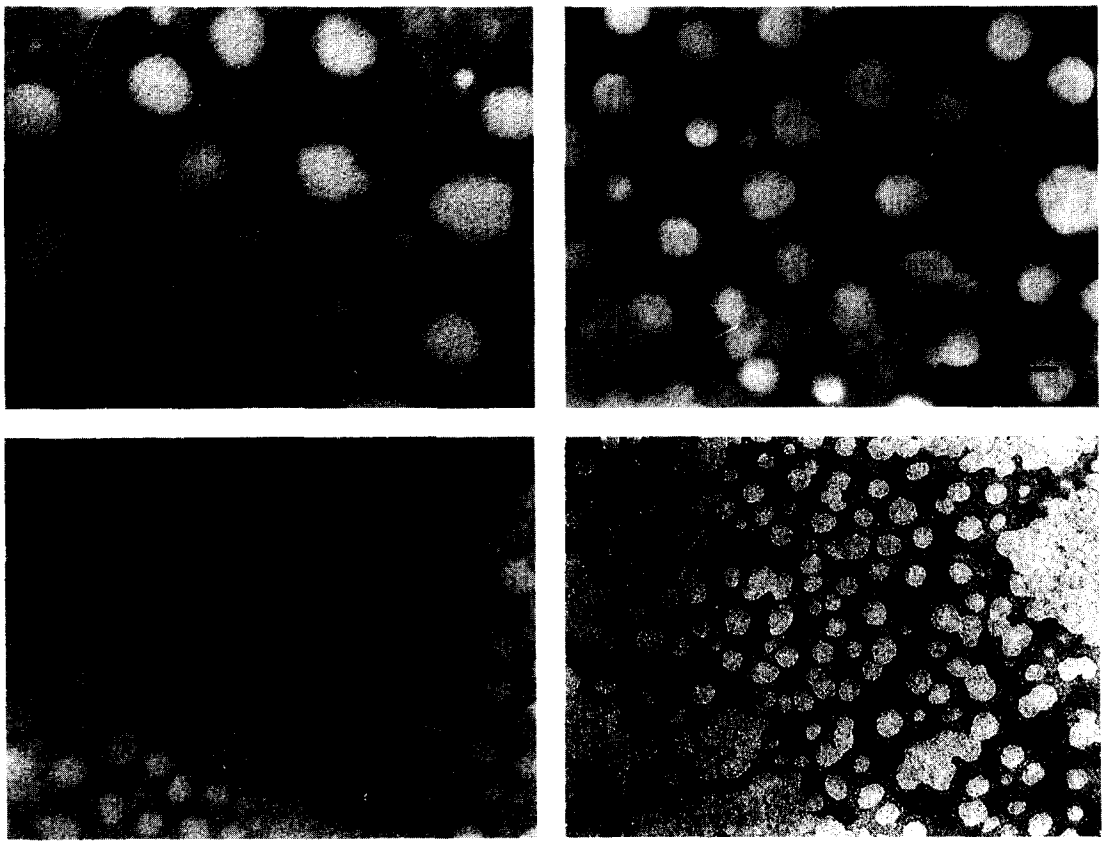

Fig. 1. Electron microphotograph of the liposomes. Magnification: 150 000 ×. The bar corresponds to 60 nm. (a) P90 egg liposomes obtained by sonication; (b) P90 soya liposomes obtained by sonication; (c) P90 egg liposomes obtained by dialysys; (d) P90 soya liposomes obtained by dialysis.

with a 2% phosphotungstic acid solution (pH 7.0). For better spreading of vesicles, the Formvar carbon-coated grids were treated with 1% bacitracin solution according to the method of Gregory and Pirie (1972).

Turbidity measurements were carried out using a Perkin Elmer LS5 spectrofluorometer with excitation and emission wavelengths both set at 600 nm.

As previously pointed out, liposomes were prepared according to two methods as detailed below.

2.1. Method A (sonication)

The appropriate amounts of phospholipids (from 40 to 160 mg) were completely dissolved in 4–5 ml of methanol. The solvent was completely removed by vacuum evaporation to form a thin film of lipids inside the vessel. 5.0 ml of the Hepes buffer were added, the mixture was gently shaken for 1 h and then sonicated, under a nitrogen stream, from six to 10 times for 5 min (with intervals of 2 min), according to the amount of phospholipid present in the formulation. The temperature was maintained at 15–20°C by means of a water bath.

When cholesterol-containing SUV were prepared, the molar ratio of phospholipid to cholesterol was always 7:3, and the same procedure was followed for the preparation of the vesicles.

2.2. Method B (dialysis)

As in method A, the appropriate amounts of phospholipids were completely dissolved in 4–5 ml of methanol. After complete evaporation of the organic solvent, 5.0 ml of 2.6×10^{-2} M sodium cholate in the Hepes buffer were added, the mixture was gently shaken for 10 min and then dialyzed for 24 h.

The same molar ratio, as that reported above, between the two components (7:3) was used for cholesterol-containing liposomes.

Based on an average molecular mass of 800 Da, the final phospholipid concentrations of the tested liposome preparations were 10, 20, 30 and 40 mM. The reproducibility of the different preparations was checked by turbidity measurements (fluctuations of $\pm 3.5\%$).

Sodium cholate concentrations were determined by means of a spectrophotometric method, as described by Fini and Zuman (1993), using a Perkin Elmer Lambda 3A spectrophotometer.

3. Results and discussion

Fig. 1 demonstrates the transmission electron microphotograph of the liposomes prepared with egg and soya phospholipids, according to the two methods. As can be observed, sonication always led to larger vesicles (80–150 and 20–140 nm for egg and soya phospholipids, respectively). When dialysis was used, SUV dimensions ranged from 25 to 50 nm for egg phospholipids and from 10 to 20 nm for soya phospholipids. The results obtained also indicated that soya liposomes were on an average smaller than egg liposomes and that, for the tested preparations, the maximum polydispersity value was found for soya vesicles prepared by sonication.

As reported above, Triton X-100 was used to study SUV-detergent interactions. Fig. 2 and 3 present R^{sol} and R^{sat} values of liposomes pre-

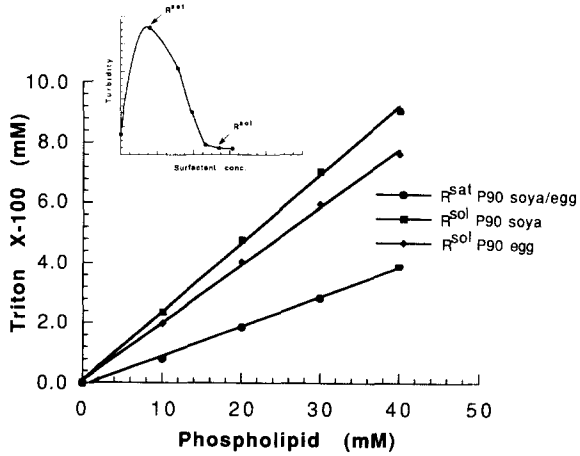

Fig. 2. R^{sol} and R^{sat} values, determined with Triton X-100, for liposomes obtained by sonication and prepared with different concentrations of P90 soya or P90 egg. (Inset) Effect of increasing surfactant concentration on the turbidity of a liposome dispersion; points corresponding to R^{sol} and R^{sat} values are indicated by an arrow.

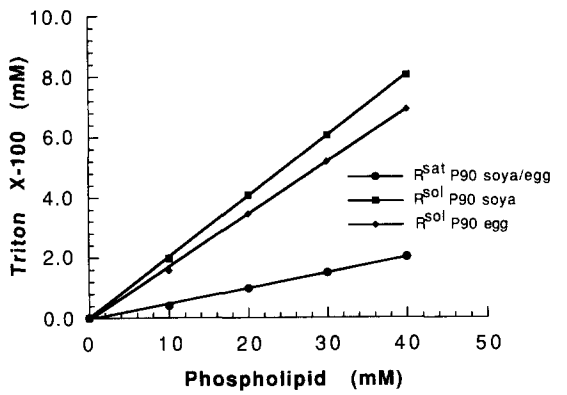

Fig. 3. R^{sol} and R^{sat} values, determined with Triton X-100, for liposomes obtained by dialysis and prepared with different concentrations of P90 soya or P90 egg.

pared with different concentrations of P90 soya and P90 egg, and obtained by sonication or by dialysis. These values were calculated from the corresponding turbidity measurements. As previously pointed out, the turbidity (inset of Fig. 2) increases, reaches a maximum value corresponding to the detergent-saturated vesicles (R^{sat}), then gradually decreases and reaches a minimum when only mixed micelles are present (R^{sol}). As can be observed, a linear relationship between phospholipid concentration and both R^{sol} and R^{sat} was obtained, furthermore, while in the case of R^{sol} different values were detected with P90 soya and with P90 egg, no difference was observed for R^{sat} values.

If we consider that the amount of surfactant needed to break down the vesicle structure (i.e., R^{sol}) can represent a significant index of the stability of the aggregated structures (Paternostre et al., 1988), the results obtained show how soya liposomes are more stable than egg liposomes; furthermore, a comparison between Fig. 2 and 3 indicates that liposomes prepared by dialysis appear to be less stable than those obtained by sonication. This lower stability cannot be related to the small amount of sodium cholate which may be still present in the vesicle structure when dialysis is employed. In fact, the same R^{sol} values were always detected with vesicle preparations obtained after 6, 12 and 24 h of dialysis (corresponding to cholate concentrations ranging from 1.0×10^{-3} after 6 h to 1.0×10^{-4} M after 1 day). Furthermore, it should be pointed out that sodium cholate, like other surfactants, can be used to break down vescicular structures; in this sense, the R^{sat} and R^{sol} values were also determined with this detergent. The data obtained indicated that again no differences in R^{sat} values were detected, while a higher cholate concentration was needed to reach the R^{sol} value in the case of soya liposomes (e.g., in the case of liposomes prepared by dialysis, for 20 mM phospholipid concentration, $R^{sat} = 6.1 \times 10^{-3}$ M for both P90 vesicle preparations, while $R^{sol} = 1.6 \times 10^{-2}$ and 2.0×10^{-2} M, respectively for egg and soya SUV).

In accordance with the electron microphotographs that show the vesicle dimensions, the different stability to detergent-induced rupture can be related to the actual size of the vesicles. In fact, smaller liposomes prepared by dialysis, due to their extreme curvature, have a greater free energy content than larger ones (Lentz et al., 1987) obtained by sonication. Of course, such an argument is not valid for a comparison among liposomes prepared with different phospholipids (e.g., egg and soya as in our case), since a difference in phospholipid composition is also indicative of a different flexibility of the aggregated structure.

Finally, it is interesting to point out that, when cholesterol, commonly used to increase liposome resistance, was also present in SUV formulations, the differences in R^{sol} values became negligible for both methods of vesicle preparation, while P90 soya liposomes still gave higher values of R^{sol} (approx. 10%) than those observed with P90 egg.

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