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Liposomes in cosmetics: Which kind of phospholipid? Which loading method?

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Synopsis

Phospholipids of different origin (egg and soya) and purity were used to prepare liposomes by sonication. Loading of these vesicles was performed by means of two different techniques using a fluorescent lipophilic model molecule. The stability of the aggregated structures was checked by addition of increasing amounts of a surfactant to the liposome dispersion. No remarkable differences were observed in either the stability in regard to surfactant-induced breakage or the loading capacity of liposomes respectively prepared with 99% pure egg phosphatidylcholine or with the vegetable phospholipid, a commercial product that had a much lower purity. The comparison of the two loading methods indicated that incorporation of the model molecule within the vesicle structure was higher when the fluorescent marker was added before sonication.

INTRODUCTION

It is well known that double-chain amphiphiles, such as phospholipids, are capable of aggregating into bilayers that assume the form of liposomes: closed spheres of different structures and dimensions that can be loaded with active ingredients. Because of these properties, liposomes are present in several pharmaceutical preparations and are largely used in cosmetics.

The origin, the extraction, and the purification method, and consequently the final composition and purity of the phospholipids used for the preparation of liposomes, can lead to dramatically different prices and at the same time to a great variety of loading capacity and stability structures (1).

The aim of this work was to compare the behavior of a 99% pure egg phosphatidylcholine (EPC) with that of a vegetable phospholipid that had a much lower purity and price (P90). In this sense it is also interesting to point out that, as far as the origin (egg or soya) is concerned, the vegetable phospholipid appears to be more appropriate for

topical applications in cosmetics and dermatology because of the high content of polyunsaturated fatty acids (2), like linoleic acid, which are particularly valuable in cosmetic preparations (1). The effects of different methods of liposome loading were also considered.

Since the main expected differences must be related to the bilayer structure of the vesicles, 1,6-diphenyl-1,3,5-hexatriene (DPH) (3) was chosen for our studies. This fluorescent probe, whose interaction with phospholipid vesicles was recently reviewed (4), is localized within the lipid bilayers, and the phospholipid phase transitions induce only very small changes in DPH excited-state interconversion.

Stability in regard to surfactant-induced breakage and loading capacity were respectively evaluated by means of turbidity measurements and fluorescence determinations of the probe incorporated or absorbed in the hydrophobic bilayer of the vesicles prepared by sonication.

MATERIALS

99% pure L-α-phosphatidylcholine from egg yolk (Sigma, type III-E, hexane solution, 100 mg/ml; and type XI-E, chloroform solution, 100 mg/ml) and 90% pure enriched soya phosphatidylcholine (Phospholipon 90, Nattermann Phospholipids GmbH) were used for vesicle preparation. Crystalline DPH was purchased from Sigma. Solutions and dispersions of this marker were prepared just before use and handled as much as possible in the dark because of the photosensitivity of DPH (4).

pH 7.5 HEPES solutions (10^{-3} M), made with freshly distilled and deaerated water, were used. Cholesterol, Triton X-100, and all other products used for the present investigation were of analytical grade. All solvents were tested for fluorescence at the wavelength of interest for our studies.

Fluorescence measurements were carried out by means of a Perkin Elmer LS5 spectrofluorometer using an excitation wavelength of 350 nm and an emission of 425 nm (slit 5/5 nm). Turbidity was evaluated with the same instrument, with excitation and emission wavelengths both set at 600 nm.

Sonication was performed with a Soniprep 150 apparatus (MSE, Crowley) equipped with a 19-mm probe, operating at 23 KHz and with an amplitude of 6 μm.

A phospholipids B test kit (Wako Chemicals GmbH) was used for quantitative determinations of these substances.

METHODS

Vesicles containing DPH were prepared according to two different techniques.

METHOD A (MIXED FILM)

The appropriate amount of phospholipid (80 mg of P90 or 800 μl of EPC solution), 5.6 mg of cholesterol, and 222 μl of a 2×10^{-4} M methanol solution of DPH were completely dissolved in 4–5 ml of methanol. The solvent was vacuum evaporated to

form a thin film of lipids and additives inside the vessel. 2.5 ml of HEPES buffer were added; the mixture was kept in the dark for 2 h, then gently shaken for 1 h and sonicated, under a nitrogen stream, for 40 min (8 times for 5 min). The temperature was maintained at 15–20°C by means of a water bath. The liposome dispersion was finally diluted 1:1 with HEPES.

METHOD B (ABSORBED FLUOROPHORE)

SUV were prepared according to the same procedure described above, but no marker was added until the final dilution. This 1:1 dilution at the end of the vesicle preparation was performed with a DPH dispersion prepared as follows: 4–5 ml of methanol were added to 222 μl of the 2×10^{-4} M methanol solution of DPH, the solvent was vacuum evaporated, 2.5 ml of HEPES were added to the residue, and the mixture was then vortexed and sonicated to obtain a homogeneous dispersion of DPH. Unmarked liposomes were kept in the dark overnight with the fluorophore dispersion. Longer times did not significantly increase the amount of absorbed DPH.

It has been pointed out (5) that sonication of phospholipid dispersions leads mainly to small unilamellar vesicles (SUV, 10–100 nm), but according to the aim of this study, actual liposome sizes were not determined.

Liposome separation from the "free" phospholipids and non-incorporated DPH was performed on 1-ml samples with Sephadex G200. Columns were eluted with HEPES and all the vesicles were collected (liposomes were eluted with the void volume and their presence was checked by means of a turbidity test) to reach a final volume of 5 ml. The phospholipids B test was performed before and after the passage through the columns in order to verify the percentage of aggregated form with respect to the total amount used. All final preparations containing the vesicles were tested for turbidity. The reproducibility of these last measurements, performed on the different preparations, indicated that the average dimensions and concentration of liposomes were to be considered as constant (e.g., for all liposomal dispersions corresponding to a phospholipid concentration of 0.3 mg/ml, turbidity = 71.8 ± 2.1).

DPH fluorescence was initially determined on intact purified liposomes in order to verify once more the reproducibility among the various preparations of the same kind. The vesicle structure was then broken by dilution (1:9) with methanol for the determination of the total amount of DPH present in the vesicles. Quantitative DPH determinations were obtained from appropriate calibration curves of the marker in methanol.

In order to study and compare the resistance of liposomes, the change in turbidity by progressive addition of a surfactant (Triton X-100) was measured (6,7).

RESULTS AND DISCUSSION

In Table I the fluorescences of DPH in the vesicle dispersion are reported for the different types of phospholipids and for the two methods of vesicle loading. In the same table the fluorescence measured when vesicles were broken with methanol is also given.

Table I
Fluorescence Values of DPH-Loaded Liposomes Prepared With Phospholipids of Different Type and Fluorescence Determined on Vesicles Broken With Methanol

Phospholipid	Method A	Method B
EPC	170.4 ± 8.5	148.5 ± 8.0
EPC (broken SUV)	47.9 ± 2.5	34.7 ± 2.0
P90	160.0 ± 8.2	144.3 ± 8.0
P90 (broken SUV)	45.5 ± 2.5	33.7 ± 2.0

Reported results represent the mean values obtained from five separate experiments. Reproducibility can be evaluated by the low range of fluorescence fluctuations in the different preparations (~5%). As it is possible to observe, only a small difference in DPH fluorescence between EPC and P90 liposomes has been detected. When compared with intact vesicles, broken liposomes in methanol always gave a much lower fluorescence because of the presence of the organic solvent (4). From the fluorescence values reported in this table, it is also possible to observe that less DPH was present in the "Method B" formulations because of the smaller amount of the probe that can be liposomally incorporated by means of this technique. The marker actually present in the vesicles was calculated from the values determined in methanol (i.e., after the breakage of the aggregated structure), where fluorescence is linearly dependent on DPH concentration. In this sense, it must also be pointed out that surfactants are often used to disaggregate liposome structures, but their presence leads to higher fluorescence values in water dispersions, gives non-linear calibration curves, and can induce fluorescence changes in the fluorophore (8–10) that can yield uncorrect or misleading results.

The phospholipid test indicated that over 95% of the initial amount of phospholipids was recovered as SUV after the passage through Sephadex; nevertheless, for a correct comparison among the different preparations, these minor variations have been considered and the percentage of entrapped or absorbed DPH was calculated according to the following expression:

$$\% \text{ DPH} = \frac{[\text{DPH}]_a}{[\text{DPH}]_b} \times K \times 100$$

where the subscripts a and b indicate the DPH concentrations (mmoles $\times$ ml^{-1}) after and before purification, respectively, and K is the ratio between phospholipid concentrations (mg/ml) before and after the passage through Sephadex. The coefficient K allows comparison of the different preparations by considering the small loss of phospholipid during purification and by correcting at the same time the dilution factor.

In Table II the percentage of directly entrapped (Method A) or absorbed DPH on empty vesicles (Method B) is given for both EPC and P90. No variations between type III-E and XI-E EPC were detected. Reported results are the average values obtained from five separate experiments.

As it can be observed from obtained results, the difference in loading capacity between EPC and P90 vesicles, although detectable, is always below 4%.

In order to compare the stability of EPC and P90 liposomes, the changes in turbidity of the vesicle dispersion by addition of increasing amounts of Triton X-100 were

Table II
Effect of the Type of Phospholipid and of the Loading Technique on the Percentage of DPH in the Vesicles

Phospholipid	% DPH in liposomes	
	Method A	Method B
EPC	74.9 ± 3.6	54.1 ± 3.0
P90	71.2 ± 3.2	52.6 ± 3.1

evaluated. The turbidity initially increased, indicating that surfactant molecules were incorporated by the vesicles; then it decreased almost linearly because of the formation of mixed micelles (9). The trend of these curves can be directly related to the stability of the aggregated structure in the form of vesicles (7). Results reported in Figure 1, which refer to several different preparations and phospholipid concentrations, indicate that no difference was observed between the two types of liposomes.

CONCLUSIONS

From an overall comparison between EPC and P90 liposomes, reported results indicate that the differences, although detectable in some cases, are never such that they support the use of the 99% pure and much more expensive product for large scale or commercial preparations.

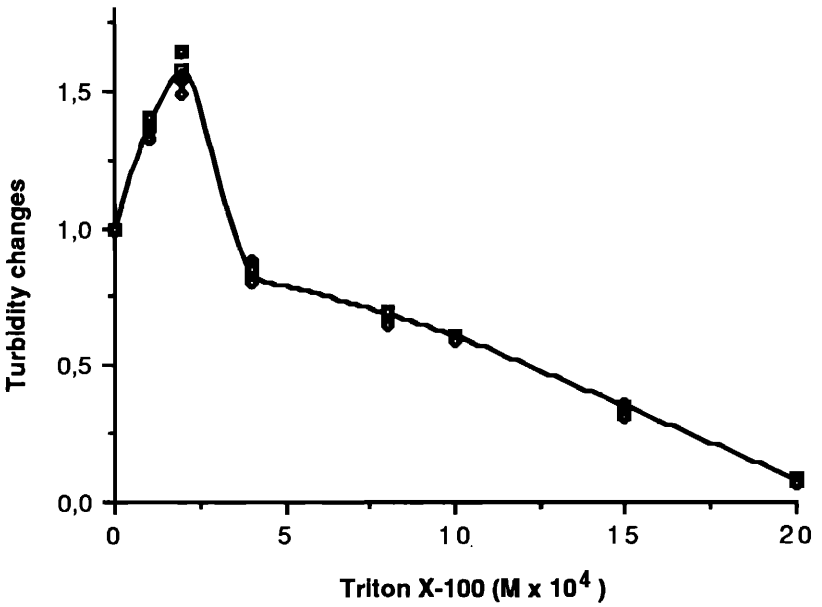

Figure 1. Effect of increasing surfactant concentration on the turbidity of liposome dispersions. Turbidity changes are expressed as the ratio between the value observed in the presence of Triton X-100 and that of the reference without surfactant. Reported experiments refer to EPC and P90 liposomes. Phospholipid concentrations were 0.30 mg/ml and 0.90 mg/ml. For the higher phospholipid concentration, abscissa values must be multiplied by 3.0.

Experimental results indicate also that the amount of lipophilic probe in the liposome structure is affected by the loading method; consequently, the possibility of incorporating hydrophobic substances in empty liposomes (that can be found directly on the market) can lead to a product that is different from that obtained when liposomes are prepared from a co-precipitated film.

REFERENCES

(1) H. Lautenschläger, J. Röding, and M. Ghyczy, The use of liposomes from soya phospholipids in cosmetics, *SÖFW*, **114**, 531–534 (1988).
(2) J. Pikul and F. A. Kummerow, Thiobarbituric acid reactive substance formation as affected by distribution of polyenoic fatty acids in individual phospholipids, *J. Agric. Food. Chem.*, **39**, 451–457 (1991).
(3) B. J. Litman and Y. Barenholz, Fluorescent probe: Diphenyhexatriene, *Methods Enzymol.*, **81**, 678–685 (1982).
(4) T. Parasassi, G. De Stasio, R. M. Rush, and E. Gratton, A photophysical model for diphenylhexatriene fluorescence decay in solvents and in phospholipid vesicles, *Biophys. J.*, **59**, 466–475 (1991).
(5) H. Ringsdorf, B. Schlarb, and J. Venzmer, Molecular architecture and function of polymeric oriented systems: Models for the study of organization, surface recognition and dynamics of biomembranes, *Angew. Chem. Int. Ed. Engl.*, 27, 113–158 (1988).
(6) K. Anzai, H. Utsumi, K. Inoue, S. Nojima, and T. Kwan, Electron spin resonance studies on the interaction between liposomal membrane and Triton X-100, *Chem. Pharm. Bull.*, **28**, 1762–1767 (1980).
(7) G. Strauss, F. Alhaique, A. Memoli, E. Santucci, and F. M. Riccieri, The stability of drug-carrying liposomes in the presence of detergents, *Polymer Preprints*, 27, 48 (1986).
(8) N. J. Turro, M. Grätzel, and A. M. Braun, Photophysical and photochemical processes in micellar systems, *Angew. Chem. Int. Ed. Engl.*, **19**, 675–696 (1980).
(9) A. K. Mathur, C. Agarwal, B. S. Pangtey, A. Sing, and B. N. Gupta, Surfactant-induced fluorescence changes in fluorescein dye, *Int. J. Cosmet. Sci.*, **10**, 213–218 (1988).
(10) Y. H. Paik and S. C. Shim, Photophysical properties of psoralens in micellar solutions, *J. Photochem. Photobiol. A*, **56**, 349–358 (1991).