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Effects of free fatty acids and transition temperature on the stability of dry liposomes

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Previous studies have shown that liposomes composed of phospholipids with low phase-transition temperatures can be stabilized in the absence of water, provided that fusion is inhibited between the vesicles during drying, and that during rehydration the phospholipids do not pass through the gel to liquid crystalline phase transition. These conditions are met by adding certain disaccharides to the vesicles before drying, which inhibit fusion and depress the transition temperature in the dry lipids. The present study shows that preservation can also be achieved with vesicles made from dipalmitoylphosphatidylcholine (DPPC), but that the retention of trapped solute by such vesicles is much less than in vesicles composed of more fluid phospholipids. Addition of free fatty acids to the vesicles before drying destabilizes them; DPPC vesicles containing 15 mol% or more of palmitic acid leaked all their contents during drying, regardless of how much of the stabilizing sugar was added. Unlike the case for more liquid phospholipids, the leakage in DPPC vesicles is due solely to fusion and not to hydration-dependent phase transitions. Addition of free fatty acids results in increased fusion, leading to leakage.

Introduction

It is well-established in the literature that liposomes [1–4], neutral biological membranes [5–7] and water-soluble proteins [6–8] can be preserved when they are dried in the presence of certain disaccharides, most notably trehalose and sucrose [1–8]. Trehalose appears to be somewhat more effective with liposomes [3], but much more effective with natural membranes [9]. Our knowledge of the effectiveness of these sugars at stabilizing liposomes composed of complex mixtures of phospholipids is rudimentary, most published experiments to date having been done with binary mixtures of fluid phospholipids, such as POPC or egg PC and PS [1–4]. That work shows that the sugars essentially completely inhibit fusion during drying [3,10]. However, inhibition of fusion alone is not sufficient to account for the preservation [3]. In addition to inhibiting fusion, there is good evidence that the sugars reduced the transition temperature (T_m) of the dry lipids [3,11–14] by as much as 90 C° [15]. The result is that the bilayers can be maintained in liquid crystalline (L_α) phase even in the absence of water (reviewed in Refs. 6 and 7). Thus, during rehydration, the bilayers dried in the presence of trehalose would not pass from gel to liquid crystalline phase. Since passage through this phase transition in hydrated bilayers results in leakage [16,17], we proposed that depression of T_m in the dry lipids, avoiding the phase transition, is a key factor in preserving them (reviewed in Refs. 6 and 7). More recently, this idea has been extended to intact cells, with similar results [18].

It follows from this suggestion that if vesicles are made from a phospholipid with high T_m, such as DPPC ($T_m = 41°C$ in excess water), the phospholipids will be in L_β phase at room temperature. When they are dried, T_m rises to as much as 120 °C [15,19,20], so they clearly remain in L_β phase during drying and subsequent rehydration. In such vesicles, the L_β to L_α transition is obviated during dehydration and rehydration. Thus, if passage through T_m is not a source of damage to dry DPPC vesicles, inhibition of fusion would be expected to be sufficient to preserve them. In the present paper we show that this is the case.

Anything that promotes fusion during drying of vesicles would be expected to lead to increased damage; one such factor is the presence of free fatty acids. There is strong evidence that free fatty acids are fusogenic in

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hydrated vesicles [21,22]. Furthermore, it has been shown by several workers that dry phospholipids can be de-esterified in intact cells [23] and pure phospholipid vesicles [24], leading to production of free fatty acids. We will show here that addition of even moderate amounts of free fatty acids to DPPC vesicles before drying them destabilizes them completely.

Materials and Methods

Lipids and preparation of vesicles. DPPC and palmitic acid were purchased from Avanti Polar Lipids (Birmingham, AL) and Sigma Biochemicals (St. Louis, MO) and used without further purification. Vesicles were prepared by sonicating multilamellar vesicles of DPPC at about 50 °C, followed by cooling to room temperature. Vesicles containing palmitic acid were prepared by dissolving weighed amounts of the dry fatty acid and DPPC together in chloroform. The solution was then dried, and multilamellar vesicles prepared by vortexing in water. The vesicles were then sonicated to clarity in a bath sonicator (Lab Industries, Hicksville, NY). The drying treatment consisted of transferring the hydrated vesicles from room temperature to liquid nitrogen, followed by lyophilization. The vesicles were rehydrated simply by adding water at room temperature.

Leakage and fusion studies. Leakage was recorded essentially as described previously [4]. Briefly, carboxyfluorescein (CF) was trapped in the aqueous interior of the vesicles during sonication. Excess CF was then removed by column chromatography. CF outside the a subsample of the vesicle preparation was recorded fluorometrically, followed by measurement of the amount inside after the vesicles were lysed by addition of Triton X-100. The remainder of the preparation was then subjected to the drying treatment, after which they were rehydrated and CF inside and outside measured. Fusion between vesicles as a result of drying was estimated by resonance energy transfer between fluorescent probes incorporated into the bilayer [10].

Differential scanning calorimetry. Thermotropic phase transitions were measured with a Hart 7707 Series differential scanning calorimeter, assisted by an IBM PC-XT data station and Hart Scientific (Provo, UT) software.

Results and Discussion

Effects of fatty acids on retention of trapped solute

The sonicated vesicles composed of DPPC alone can be stabilized to the extent that they retain as much as 70% of trapped CF when they are dried in the presence of 3.0 g sucrose/g lipid (Fig. 1). By contrast, somewhat larger vesicles of more fluid lipids will retain as much as 100% of their contents when dried in the presence of

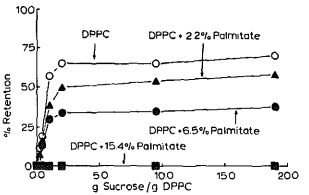

Fig. 1. Retention of trapped carboxyfluorescein (CF) by rehydrated vesicles of DPPC containing the indicated amounts of palmitic acid and previously dried in the presence of the indicated amounts of sucrose.

similar amounts of trehalose [1–4]. When the palmitic acid was added to the DPPC vesicles, retention of trapped CF declined (Fig. 1). With as much as 15 mol% palmitate present, the vesicles leaked all their contents during drying, regardless of how much sucrose was added. The effect of addition of the fatty acid was clearly to reduce retention, and it does so in direct proportion to the amount added (Fig. 2). In the following paragraphs we investigate the mechanism by which the fatty acids disrupt the stabilizing effect of sucrose on the dry vesicles.

Effects of fatty acids on phase transitions

Since disaccharides have been shown to have a marked effect on the main phase transition in dry DPPC [11,14,15], it would seem likely a priori that depression of T_m might be involved in preserving dry vesicles made from this lipid. However, the large effects of sugars on T_m of dry DPPC that we [11,15] and others [12–14] have described previously are obtained under specialized conditions; the DPPC and sugar must be dried either from organic solvents [11] or at elevated temperatures well above the T_m for hydrated DPPC

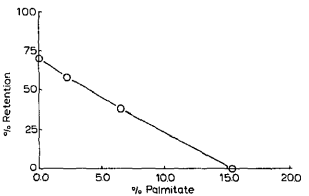

Fig. 2. The relationship between maximal retention of trapped CF and palmitic acid content in vesicles of DPPC that had been previously dried.

[14,15]. Under these conditions, T_m is depressed by as much as 90 C° [15]. However, when DPPC is dried at low temperatures, as in the present experiments, T_m is depressed only by a relatively small amount, even in the presence of large amounts of sugar [14,15]. As a result, it will not pass through the gel to liquid crystalline transition during rehydration. Nevertheless, there is some possibility that partial depression of T_m might be involved for unsuspected reasons. It follows that the fatty acids might interfere with the interaction between the sucrose and DPPC and this interference could explain the disruptive effect of the palmitic acid. We tested this hypothesis, using DSC to record phase transitions in dry DPPC. The results show that the vesicles dried without sucrose or fatty acid have a T_m approaching 90°C (Fig. 3), which is close to T_m for DPPC monohydrate [19,20]. In samples protected more carefully from contact with water vapor T_m is as high as 120°C [15,19,20]. In vesicles dried with sucrose but without palmitic acid, T_m falls to 47°C (Fig. 3). In the presence of the fatty acid, the main transition temperature does not change significantly (Fig. 3), so we conclude that this is not the mechanism by which it interferes with the preservation by sucrose.

The DSC scans shown in Fig. 3 suggest that there is a phase separation of the palmitic acid and DPPC during drying; in the samples containing palmitic acid, two melting endotherms are seen: the main transition at 47°C, assigned to the DPPC, and an endotherm at about 40°C, assigned to the palmitic acid. In the hydrated mixture prior to drying there is only a single endotherm, at about 41°C. Thus, unlike mixtures of

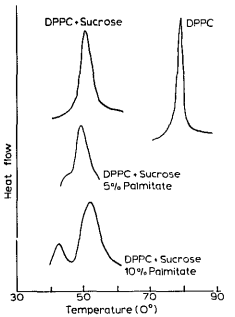

Fig. 3. Differential-scanning calorimetry scans of dry DPPC vesicles containing the indicated amounts of palmitic acid. Sucrose content of the samples containing sucrose was 5.0 g sucrose/g DPPC in every case.

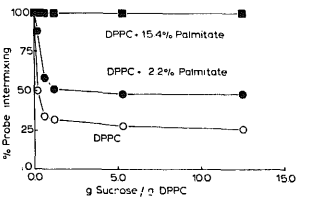

Fig. 4. Intermixing between donor and acceptor fluorescent probes in rehydrated DPPC vesicles containing the indicated amounts of palmitic acid and previously dried in the presence of the indicated amounts of sucrose.

phospholipids, which do not show phase separations during drying in the presence of high concentrations of disaccharides [3], DPPC and palmitic acid appear to undergo dehydration-induced phase separation, even in the presence of large amounts of the sugar. The mechanism by which the phase separation is likely to occur during dehydration has been described elsewhere [6,7,25].

Effects of fatty acids on fusion

In vesicles dried without addition of palmitic acid, probe intermixing declines to about 25% as sucrose content is increased (Fig. 4). With further elevation in sucrose content, probe intermixing does not decline further, which may account for the leakage of a significant proportion of the contents of the vesicles (cf. Fig. 1). With the addition of free fatty acid, probe intermixing is increased; in the presence of 15.4 mol% palmitic acid, probe intermixing remains at 100%, even at the highest sucrose content (Fig. 4). This result is in agreement with previous findings that free fatty acids are fusogenic for natural membranes and phospholipid vesicles [21,22] in the presence of excess water.

The relationship between fusion and leakage

The probe-intermixing experiments shown in Fig. 4 were done with equimolar concentrations of vesicles containing donor and acceptor probes. Fusion between vesicles containing acceptor probes is not detected by this assay, nor is fusion between vesicles containing the donor probes. Since the donor and acceptor vesicles are present in equal proportions, these homotypic fusions would be expected to occur with a probability of 0.50 and the heterotypic (donor–acceptor) fusions occur with the same probability. Thus, probe intermixing of 50% represents a single fusion event per vesicle, on average, a statistic that is illuminating concerning the relationship between fusion and leakage. When retention of

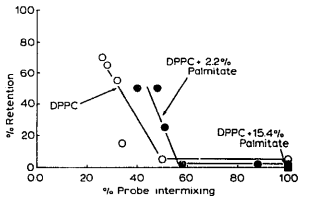

Fig. 5. The relationship between retention of trapped CF by DPPC vesicles and probe intermixing following a dehydration-rehydration cycle. Similar data are shown for vesicles containing the indicated amounts of palmitic acid.

trapped CF was plotted as a function of probe intermixing, Fig. 5 was obtained. In the absence of palmitic acid, retention remains at essentially zero until probe intermixing declines below about 50%. In fact, the line segment representing the increase in retention (fit by least squares) has an intersection with the abscissa at 51% probe intermixing. Thus, retention of trapped solute remains at 0% until the frequency of fusion declines below one fusion event per vesicle, after which retention and fusion are linearly and inversely related (Fig. 5). Similar results were obtained with the samples containing palmitic acid. Although retention is depressed relative to the levels seen in samples without the fatty acid, in those with 2.2% palmitic acid the intersection with the abscissa is at 55% probe intermixing. In the presence of 15.4% palmitic acid, probe intermixing is at 100% and retention remains at 0% under all conditions.

Conclusions

We conclude from these data that the primary event leading to leakage of trapped solute from dried vesicles composed of DPPC is fusion induced by dehydration. It appears that even though disaccharides are capable of depressing T_m in dry DPPC, phase transitions and their depression by sugars play little or no role in leakage seen as a result of dehydration in such vesicles. This finding contrasts sharply with previous results showing that depression of T_m is required for preservation of vesicles composed of more fluid lipids that pass through the phase transition during dehydration-rehydration cycles. We now add the corollary that in vesicles that do not pass through T_m during dehydration or rehydration cycles, it is necessary only to prevent their fusion to achieve maximal stability. Finally, we conclude that addition of free fatty acids leads to fusion during dehydration, as a consequence of which the vesicles leak their contents.

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