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STABILIZATION OF LARGE MULTILAMELLAR LIPOSOMES BY HUMAN SERUM IN VITRO

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The leakage of 5,6-carboxyfluorescein from large multilamellar liposomes prepared from dipalmitoylphosphatidylcholine (without or with cholesterol) was investigated in vitro in the presence of human serum. Below the phospholipid phase transition temperature, the rate of dye release is retarted 3–8-fold in the presence of up to 25% human serum in the incubation medium, as compared to the release in isotonic phosphate-buffered saline. This effect is significantly augmented by incorporation of 50 mol% cholesterol into the lipid bilayer. At and above the phase transition temperature, the initial rapid dye leakage in the presence of serum is followed by a slow long-term release. Incubation of the liposomes with serum is assumed to result in the association of serum proteins with the outermost lipid bilayer which in turn will lead to their stabilization, while the inner lamellae are not immediately accessible to the serum proteins. The permeability of the outer protein-rich lipid bilayer appears to be restricted, as concluded from the decreased dye release in the presence of serum. Massive leakage from multilamellar liposomes appears to be primarily due to bilayer defects occurring in the lipid transition region rather than being caused by protein-lipid interactions. The results of our in vitro experiments are discussed in terms of the potential usefulness of multilamellar liposomes as drug carriers in vivo for local and topical applications.

Introduction

The prospect of novel applications for large multilamellar liposomes has recently renewed interest in their potential as drug carriers in vivo. As an alternate approach to existing technologies [1], these liposomes might serve as biodegradable depot systems with controlled release properties in lotion-, ointment-, or similar formulations for local or topical administration [2–4]. To optimize the potential of liposomal carriers, it is important to characterize their stability in terms of controlled release of their contents in vitro, simulating physiological conditions in vivo. The stability of small unilamellar liposomes in vitro and in vivo has been thoroughly investigated [5–8]. However, only a limited number of studies is available dealing with large multilamellar liposomes. Scherphof and coworkers [9] have demonstrated the destabilizing action of serum proteins, in particular of high-density lipoproteins. The role of lipid phase transitions in accentuating serum-induced dissolutions of multilamellar liposomes has been established [10,11]. Cholesterol was shown to increase liposomal stability by reducing interactions between lipids and serum proteins [9].

Using cholesterol-free and cholesterol-rich mul-

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tilamellar liposomes prepared from dipalmitoylphosphatidylcholine and human serum as a model for biological fluids, we demonstrate in this communication, that the release of contents of this type of carrier can be effectively controlled by the lipid composition and the physical state of the lipid bilayer, and that leakage in vitro can significantly be retarded in the presence of human serum.

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine was obtained from Fluka (Basel, Switzerland) and used without further purification. Cholesterol from Merck (Darmstadt, F.R.G.) was recrystallized twice from ethanol. 5,6-Carboxyfluorescein, purchased from Eastman-Kodak, was purified as previously described [12]. [^{3}H]Inulin was purchased from New England Nuclear. Large multilamellar liposomes were prepared following standard procedures [13,14]: 2 μmol dipalmitoylphosphatidylcholine, or when indicated, 1 μmol dipalmitoylphosphatidylcholine and 1 μmol cholesterol were dissolved in chloroform/methanol (1 : 1, v/v); the organic solvent was completely removed under vacuum in a Rotavapor. 2 ml of a 100 mM carboxyfluorescein solution (in 10 mM Tris, pH 7.5) were added, the round-bottom flask was sealed under N_2 and placed in a shaking water bath at 50 °C (120 strokes/min) for 24 h, to allow the formation of fully hydrated bilayers. The size-distribution of the resulting large multilamellar liposomes (termed 'liposomes' in the text) was heterogeneous, ranging from 0.1–5 μm in diameter, as observed by negative staining and freeze-fracture electron microscopy. Immediately prior to the experiments, nonentrapped carboxyfluorescein was removed by passing aliquots of the liposome suspension through a Sephadex G-50 column (0.5 × 15 cm) equilibrated with 150 mM NaCl/10 mM Tris (pH 7.5). The liposomes, eluting in the void volume retained their previous size distribution. Typically, 100 nmol purified liposomes (determined by phosphate analysis [15]) were added to 2 ml of the various buffers and the carboxyfluorescein fluorescence, $F(t)$, was measured in certain time intervals in a SPEX-Fluorolog-Spectrofluorometer ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 520$ nm, 2 nm slit-width). All experiments were performed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing various amounts of human serum (outdated serum, obtained from Kaplan Hospital, Rehovot, heat-aggregated at 55 °C for 30 min and adjusted to pH 7.5 ± 0.1). Upon termination of the experiments, 20 μl of 10% (v/v) sodium deoxycholate in 10 mM Tris (pH 7.5) were added to release the contents of the vesicles and to obtain the total fluorescence F_T. The fluorescence values $F(t)$ and F_T, measured in serum-containing buffer were corrected for serum-induced quenching, as previously described [16]. In the presence of serum, complete disruption of the multilamellar liposomes by the detergent was not an instantaneous process, as in pure buffer, but could last over several hours depending on the serum concentration and the temperature [12]. We therefore regarded the detergent-solubilization of the vesicles in the presence of serum as completed, when the corrected F_T values had reached those of vesicles incubated in serum-free buffer. Release (R) was calculated as $(F(t)/F_T) \times 100$. Dye latency (L) was expressed as $L = 100 - R$. The half-times of long-term carboxyfluorescein release were calculated from the linear portions (after 60 min of incubation) of semilogarithmic plots of L vs. time [16]. If not stated otherwise, all experiments were performed at least twice in quadruplicates.

Results

The half-times, $T_{1/2}$, for dye release from large multilamellar liposomes under various incubation conditions were obtained as described in Materials and Methods. Shown in Fig. 1 are half-times for the long-term carboxyfluorescein release, $T_{1/2}$, as a function of the serum concentration at various incubation temperatures. At 25 °C and at 37 °C, the retarding effect of cholesterol on carboxyfluorescein release in the absence of serum was only marginal. In the presence of serum, however, cholesterol-rich liposomes released their contents up to 1 order of magnitude slower, than those composed of the pure phospholipid. The dependence of $T_{1/2}$ on the serum concentration was a function of the temperature. At $T = 25$ °C, i.e., below the transition temperature of dipalmitoylphosphatidylcholine, the increase in the

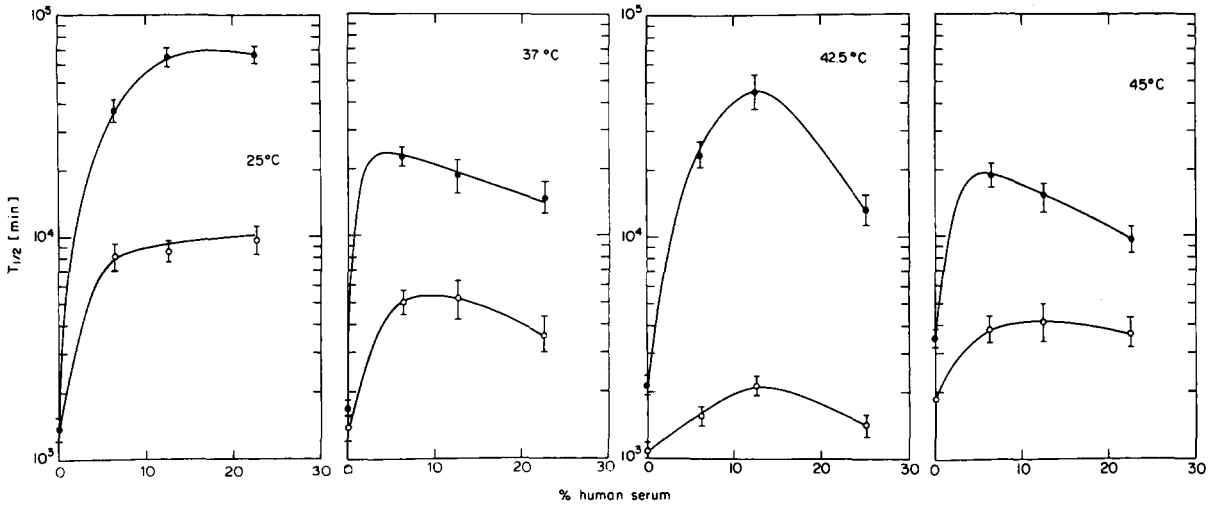

Fig. 1. Half-times of the long-term carboxyfluorescein release ($T_{1/2}$) from cholesterol-free (○) and cholesterol-rich liposomes (●) as a function of the concentration of human serum in the incubation medium (phosphate-buffered saline, pH 7.5, temperatures as indicated). $T_{1/2}$ was calculated as indicated in Materials and Methods. Data represent means ± S.D. from at least two independent experiments, each performed in quadruplicates.

serum contents in the buffer resulted in a saturable increase in $T_{1/2}$ (Fig. 1); this effect was significantly enhanced by including 50 mol% cholesterol into the lipid mixture. At higher temperatures, biphasic $T_{1/2}$ vs. serum concentration plots were obtained, the shape of which depended o the temperature. Maximal serum-induced increases in $T_{1/2}$ were observed below the phase transition temperature of dipalmitoylphosphatidylcholine; for cholesterol-free liposomes $T_{1/2}$ increased approx. 8-fold and for cholesterol-rich liposomes, $T_{1/2}$ was increased up to about 50-fold. In Fig. 2, the half-

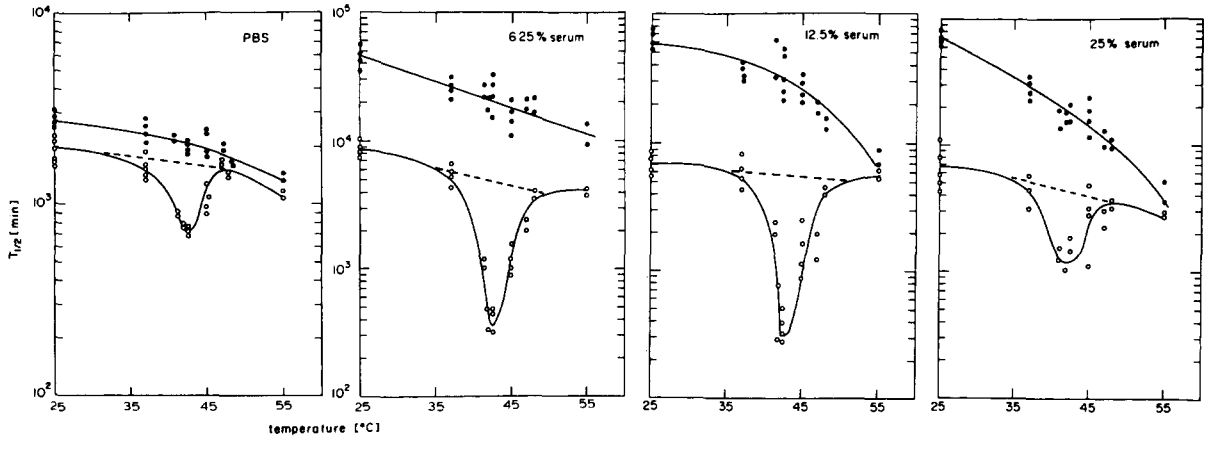

Fig. 2. Temperature dependence of the half-times of the long-term carboxyfluorescein release from cholesterol-free (○) and cholesterol-rich liposomes (●). Cumulative data. Each point represents the mean of one experiment performed in quadruplicates. For clarity, error bars have been omitted. The dotted lines connecting the pre- and the posttransition values of the half-times of carboxyfluorescein release from cholesterol-free liposomes are 'imaginary baselines' used to evaluate the extent of the transition-induced increase in carboxyfluorescein leakage. PBS, phosphate-buffered saline.

times of long-term carboxyfluorescein release are plotted against the temperature. Each plot contains the cumulative data for liposomes composed of dipalmitoylphosphatidylcholine and of dipalmitoylphosphatidylcholine/cholesterol and incubated in phosphate-buffered saline in the absence of serum and in buffer containing 6.25, 12.5 and 25% human serum, respectively. The minimum in $T_{1/2}$ found at 42.5 °C for cholesterol-free liposomes indicates maximal carboxyfluorescein-leakage at the phase transition temperature of pure dipalmitoylphosphatidylcholine (approx. 42 °C). This augmented carboxyfluorescein release at the phase transition temperature is further enhanced in the presence of 6.25 and 12.5% serum 14-fold and 20-fold, respectively, as compared to the corresponding values on 'imaginary base-lines' connecting the pre- and posttransition data. However, in the presence of 25% serum, carboxyfluorescein leakage at the phase transition temperature is augmented only 3.5-fold. Upon inclusion of cholesterol into the bilayer membranes, carboxyfluorescein release is enhanced with increasing temperature without any transition-like peak around 42.5 °C.

Initial carboxyfluorescein loss, measured within 1 min upon incubation in the different aqueous media, depends mainly on the temperature and lipid composition and less on the presence of serum proteins (Fig. 3). In phosphate-buffered saline below and above the transition region, it was only marginal (less than 10%), but approaches 40% of the total liposome contents at the transition temperature for liposomes composed of pure dipalmitoylphosphatidylcholine. Cholesterol-rich liposomes, lost markedly less of their contents in the first minute of incubation than cholesterol-free liposomes. Initial carboxyfluorescein release from cholesterol-rich liposomes was a monotonous function of the incubation temperature. A qualitatively similar picture was obtained when incubating these liposomes in buffer containing 25% serum (not shown). During prolonged incubation (5 h) in serum-free buffer, cholesterol-free liposomes lost between 5 and 75% of their contents, depending on the incubation temperature. Increasing the serum contents resulted in a significant stabilization of these liposomes. Thus, in buffer containing 25% serum, carboxyfluorescein leakage was reduced to less than 10% (at 25 and 37 °C) and to

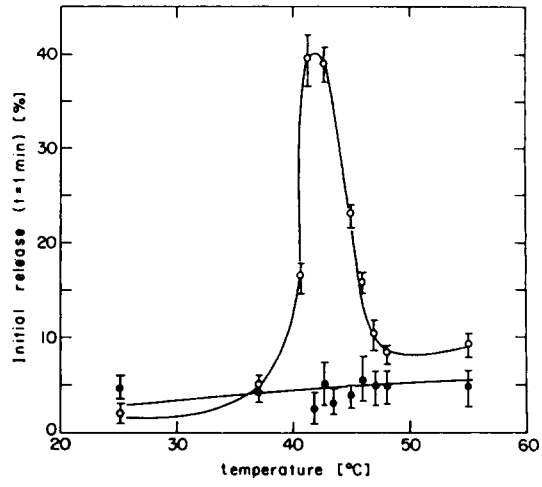

Fig. 3. Temperature dependence of the initial release of carboxyfluorescein from liposomes incubated in phosphate-buffered saline. The initial release values (% of the total contents) were measured after 1 min incubation of cholesterol-free (○) and of cholesterol-rich (●) liposomes. The data represent mean ± S.D. from two independent experiments performed in quadruplicates.

40–50% (at 42.5 and 45 °C), respectively (not shown). The loss of contents from cholesterol-rich liposomes during a 5-h incubation period below the lipid phase transition temperature decreased from about 10% in buffer to about 2.3% in buffer containing 10–25% serum (Fig. 4). When incubated for 5 h at and above the phase transition temperature these liposomes lost between 15 and 30% of their contents. Minimal long-term leakage was observed in buffer containing about 10% serum.

In addition to the data just presented, we report two further experimental observations on the release of the contents of large multilamellar liposomes:

(1) To verify our results, obtained with carboxyfluorescein, we repeated a few experiments using [^{3}H]inulin as the aqueous space marker. For all parameters checked (cholesterol contents, presence of serum in the buffer, temperature), the long-term release of the liposome contents determined by dialysis techniques was within ± 10% comparable to that obtained by the carboxyfluorescein assay.

(2) To determine their stability over an extended period of time, liposome suspensions were sealed under N_2 right after preparation, prior to

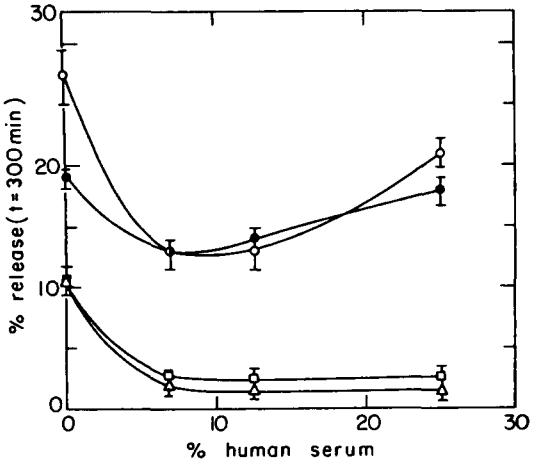

Fig. 4. Long-term carboxyfluorescein release from cholesterol-rich liposomes as a function of the serum concentration in the incubation medium. Liposomes were incubated at 25 (△), 37 (□), 42.5 (●) and 47 °C (○) in buffer containing 0–25% human serum. The amount of carboxyfluorescein released (% of the total) was determined after 300 min incubation as indicated in Materials and Methods. Data represent mean ± S.D. from two independent experiments performed in quadruplicates.

removal of nonentrapped carboxyfluorescein and stored in the dark at 4°C for up to 4 months. During this period, aliquots were removed once a week and, after separation from free carboxyfluorescein, the release of the liposomal contents was measured at 37 °C, as described. When proper care was taken, to work from the onset under sterile conditions, no significant change was found in the release characteristics of the liposomes, as compared to the results mentioned above, indicating a considerable shelf-life for these liposome formulations.

Discussion

In the present work, the stability of large multilamellar liposomes in vitro was assessed form the latency of entrapped carboxyfluorescein [5,6,8,16]. This technique allows continuous monitoring of the leakage of vesicle contents, from the onset of the incubation in different media and is more convenient in observing short-term (less than 10 min) leakage than radiotracer methods. However, our data on long-term carboxyfluorescein release were validated by dialysis experiments with liposomes containing [^{3}H]inulin as marker for the aqueous space.

Our results confirm previous findings that the physical state of the lipid membranes is a major factor in determining liposome integrity, in terms of leakage of entrapped material and/or dissolution of lipid membranes by serum proteins [9,10]. Maximal carboxyfluorescein leakage from cholesterol-free liposomes is observed at the phase transition temperature for pure dipalmitoylphosphatidylcholine, i.e., at approx. 42 °C [12,13] (Fig. 2). The increase in membrane permeability in the vicinity of the phase transition is accentuated in the presence of about 10% serum in the incubation medium, as recently reported by Yatvin and coworkers [17].

The major outcome of this study, reported here for the first time, is the protecting effect of serum proteins on the loss of contents from large multilamellar liposomes. The stabilization of the lipid membranes, accentuated in cholesterol-rich liposomes at low serum concentrations (about 10%) and below the phase transition temperature of the phospholipids, may partially reflect restricted interactions of serum proteins with lipid-bilayer membranes in the gel state [18]. It has been previously noted, that the leakage of calcein, a fluorescent hydrophilic marker similar to carboxylfluorescein, is decreased by incubating small unilamellar liposomes composed of distearylphosphatidylcholine in serum-containing buffer below the lipid phase transition temperature [19].

The long-term stabilization of multilamellar liposomes, in the presence of serum is presumably due to the temperature- and cholesterol-dependent association and/or interaction of serum proteins with the outermost lipid bilayer. Supportive evidence for this notion comes from recent experiments which indicate that the dependence of carboxyfluorescein release from multilamellar liposomes on the temperature and on the cholesterol contents is similar to the pattern of loss of di[^{3}H]palmitoylphosphatidylcholine from these liposomes to serum proteins (unpublished data). Weinstein et al. [20] recently reported that the incubation of unilamellar dipalmitoylphosphatidylcholine liposomes with serum proteins below the lipid phase transition did not induce rapid carboxyfluorescein leakage. In line with these ob-

servations, we found a significant increase in the half-time of the long-term carboxyfluorescein release from large multilamellar liposomes, indicating that (a) serum proteins do associate with the outermost lipid bilayer below the phase transition and (b) this association results in decreased membrane permeability. In our experiments, maximal protein-induced stabilization of the outer bilayer leaflets was obtained at low serum concentrations (about 10%). Further increase in the serum concentration did not modify the leakage of the dye from the liposomes at 25 °C, indicating a limited and saturable association of serum proteins with dipalmitoylphosphatidylcholine below the phase transition temperature [20]. Upon incubation at the phase transition temperature, liposome-associated proteins will penetrate into the outer bilayer, due to enhanced lipid-protein interactions [20], and cause the release of the contents of the first inter-bilayer aqueous space, similar to the serum-induced leakage of unilamellar liposomes [7,19]. Subsequently, the reduced permeability of the outermost bilayer will be the rate-limiting step for carboxyfluorescein leakage even at or above the phase transition temperature when the serum proteins can gradually penetrate also to the inner bilayers, as inferred from the increased leakiness of the liposomes at higher serum concentration (Fig. 2). Noteworthy in this context are recent findings of Kirby and Gregoriadis [21]. While studying plasma-induced permeabilization of small unilamellar liposomes, these authors investigated also the entry of carboxyfluorescein into cholesterol-poor and cholesterol-rich egg phosphatidylcholine liposomes. They found that dye was taken up into empty liposomes and exhibited in the presence of full mouse plasma a significantly higher latency than in phosphate-buffered saline alone. This plasma-induced stabilization of the small egg phosphatidylcholine liposomes was maximal (about 40%) for cholesterol-free liposomes and might have been caused by a similar mechanism of initial perturbance (leading to dye uptake) and subsequent stabilization (enhanced latency) of the lipid bilayer membranes upon interaction with plasma proteins as reported here.

Beyond a physicochemical interest for in vitro studies, our results may be significant for designing proper liposomal carrier systems in vivo. Large multilamellar liposomes were recently suggested as slow-releasing biodegradable depot systems in local and/or topical applications, e.g., in the respiratory tract, in the eye, in the joints, as suppository, or in ointment-forms (for a recent review, see Ref. 14). At all these sites of applications, body fluids are present, the electrophoretic patterns of which are quite similar to that of plasma [22]. Therefore, it seems reasonable to use human serum as a model for biological fluids in general. Furthermore, the use of 25% serum seems to be justified, since in line with previous observations [7], preliminary experiments showed that more than 90% of the effects, observable in full serum, were found in 25% serum (unpublished results). For the novel liposomal formulations, two major issues have to be addressed: (1) Prolonged retention of intact carriers at the site of administration and controlled release of their contents. Recent studies indicate that site retention and the stability of liposomal carriers in vivo can be augmented using synthetic lipids [23,24] and/or incorporating synthetic glycolipid conjugates into the bilayer membranes [25,26]. The use of synthetic yet biodegradable phospholipids seems appropriate for several reasons. Synthesis of dipalmitoylphosphatidylcholine can decrease the batch variability encountered in samples of biological origin. Furthermore, the shelf-life of liposome formulations prepared from synthetic lipids can approach commercially relevant periods as, e.g., shown in this work.

The problem of liposome retention at the desired site of action is closely related to the question of controlled drug release. It makes no sense to formulate extremely stable liposomes which are removed from their target sites before they can release significant portions of their contents, or vice versa. Therefore, the proper choice of the constituent lipids (e.g., length of their fatty acid chains, their headgroup composition and cholesterol contents) will ultimately depend on the desired retention and stability of the liposomes. As shown in this work, the rate of drug release in vitro can be modulated with sufficient accuracy, ranging from a few minutes to many hours and days.

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