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Stability of association of 1-*O*-octadecyl-2-*O*-methyl-sn-glycero-3-phosphocholine with liposomes is composition dependent

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Abstract

The ether lipid, 1-*O*-octadecyl-2-*O*-methyl-sn-glycero-3-phosphocholine (ET-18-OCH_3), has anticancer activity, but it has serious side-effects, including hemolysis, which prevent its optimal use. We surmised if ET-18-OCH_3 could be stably associated with liposomes, less free ET-18-OCH_3 would be available for lytic interaction with red cells. Liposome composition variables investigated included acyl chain saturation, phospholipid head group and mole ratio of Chol and ET-18-OCH_3. It was found that attenuation of hemolysis was strongly liposome composition dependent. Some ET-18-OCH_3 liposome compositions were minimally hemolytic. For example, whereas the HI_5 (drug concentration required to cause 5% human red cell lysis) was 5–6 μM for free ET-18-OCH_3, it was approximately 250 μM for DOPC (dioleoylphosphatidylcholine):Chol (cholesterol):DOPE-GA (glutaric acid derivatized DOPE):ET-18-OCH_3, (4:3:1:2) and 640 μM for DOPE (dioleyolphosphatidylethanolamine):Chol:DOPE-GA:ET-18-OCH_3 (4:3:1:2) liposomes. Efflux of carboxyfluorescein (CF) from liposomes and Langmuir trough determinations of mean molecular area of lipids in monolayers (MMAM) were used as indicators of membrane packing and stability. Incorporation of ET-18-OCH_3 in liposomes reduced the MMAM. Reduction in CF permeation was correlated with reduction in hemolysis. The most stable liposomes included components, such as cholesterol, DOPC and DOPE, which have complementary shapes to ET-18-OCH_3. © 1997 Elsevier Science B.V.

Keywords: Ether lipid; Liposome; Hemolysis; Molecular packing

1. Introduction

The ether lipid, 1-*O*-octadecyl-2-*O*-methyl-sn-glycero-3-phosphocholine (ET-18-OCH_3) is selectively cytotoxic to cancer cells compared to normal cells in vitro [1] and inhibits the growth of murine and human tumors in experimental systems [2,3]. Its clinical use by the i.v. route has been hampered, however, by gastrointestinal and other toxicities, including hemolysis [2–4].

We postulated that stable association of ET-18-OCH_3 with liposomes would greatly reduce its availability to interact with red cells, thereby reducing hemolysis and perhaps other unwanted side effects. It has been shown that the in vivo toxicity of various drugs can be markedly reduced by stably associating them with liposomes [5]. Several liposome formula-

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tions are in advanced clinical trials for cancer [6] and infectious diseases [7] and some have received marketing approval [8]. Although ET-18-OCH_3 has been incorporated in liposomes previously [9], no systematic study of how it interacts with various lipid compositions has been reported. Recently a 1:1.5 ET-18-OCH_3:Chol assembly was shown to have quite favorable packing properties [10]. Here, liposome formulations of ET-18-OCH_3 and cholesterol plus other components varying with respect to phospholipid saturation, head group and charge were compared using a hemolysis assay as an indicator of the availability of ET-18-OCH_3 to interact with red cell membranes. In order to more fully understand the basis for the observed effects, the permeation of carboxyfluorescein from ET-18-OCH_3 liposomes and the effect of ET-18-OCH_3 on lipid mean molecular surface area was also investigated for some formulations.

2. Materials and methods

2.1. Chemicals

1-*O*-Octadecyl-2-*O*-methyl-sn-glycero-3-phosphocholine (ET-18-OCH_3, Edelfosine), DOPC, DOPE, egg phosphatidylcholine (EPC), distearoyl phosphatidylcholine (DSPC), glutaric acid derivatized dipalmitoylphosphatidylethanolamine (DPPE-GA), glutaric acid derivatized palmitoyloleylphosphatidylethanolamine (POPE-GA), dipalmitoylphosphatidylglycerol (DPPG) and DOPE-GA were obtained from Avanti Polar Lipids (Alabaster, Alabama) except where otherwise noted. Chol was purchased from the Sigma Chemical Company, USA. Phosphate buffered saline (PBS) was purchased from Gibco BRL Life Technologies (Grand Island, New York). Human serum was obtained from Sigma Chemical (St. Louis, MO). All lipids and reagents were of the highest purity available.

2.2. Preparation of liposomes

ET-18-OCH_3 liposomes and non-ET-18-OCH_3-containing (sham) liposomes were prepared by the solvent evaporation method [11]. ET-18-OCH_3 and lipids were dissolved in chloroform to give different molar lipid ratios of phospholipid:Chol:ET-18-OCH_3 with or without 10 mol% DPPE-GA, POPE-GA or DOPE-GA. The compositions investigated are given in Table 1. The organic solvent was initially removed by rotary evaporation at 45°C, using 500 ml round bottom flasks. The thin film, containing up to 120 mg lipid, was then dried for at least 18 h at less than 1 Torr (Edwards Pump, Crawley, Sussex, England). The film was hydrated with PBS for 1 h above the transition temperature (T_m) of the lipid, forming liposome suspensions at 5–10 mg/ml ET-18-OCH_3. In initial studies the liposomes were then extruded directly. In later studies the liposomes were frozen in liquid nitrogen and thawed at 40°C for 10 cycles and then extruded 10 times through 0.1 μm double stacked Nucleopore filters using an extruder device (Lipex Biomembranes Vancouver, BC, Canada) and were examined by light microscopy to check for possible aggregation. Some preparations were analyzed for chemical composition by reverse phase HPLC (Inertsil ODS2 150 × 4.6 mm column) with an evaporative-light scattering detector using 4% water in methanol as the mobile phase. The size distributions of liposome preparations were determined using a Nicomp Model 370 Particle Sizer system (Santa Barbara, California).

2.3. Electron microscopy

For freeze-fracture electron microscopy, 1–3 μl of sample without cryoprotectant was placed between a pair of Balzers copper double replicating holders and frozen from room temperature in liquid propane [12]. A Balzers BAF 400 freeze-fracture device was used. Replicas were cleaned overnight in 5% hypochlorite (commercial bleach), washed in distilled water, mounted on 300 mesh grids and viewed with a Philips 300 TEM.

2.4. Hemolysis

Lysis of human red blood cells (RBC) by free or ET-18-OCH_3-liposomes was quantitated by hemoglobin release into buffer [13]. Various concentrations (3 μM–2 mM) of free and ET-18-OCH_3-liposomal, or ET-18-OCH_3 free liposomes (1:1 v/v, blood:formulation, serially diluted) were incubated with 4% RBC (washed in PBS, 3 ×) at 37°C for 20

h. The release of hemoglobin from an equal number of RBC by hypotonic lysis in water was taken as 100% positive control. Incubation with PBS served as a negative control. After 20 h of incubation, the samples were centrifuged at 2000 × *g* for 10 min and the absorbance of the supernatant was measured at 550 nm (hemoglobin). The concentrations of ET-18-OCH_3 (μM) required to cause 5% hemolysis (HI_5) was calculated for each liposome composition investigated by interpolating between the concentrations above and below 5% hemolysis.

2.5. Carboxyfluorescein permeability

Liposomes were prepared, as described above, in the presence of 0.1 M 5(6)-carboxyfluorescein (CF) (Molecular Probes, Eugene, OR) in PBS. CF containing liposomes were separated from non-liposome associated CF using a Sephadex G50 column at room temperature. CF liposomes were then added to PBS only, or PBS containing 0.5% serum equilibrated at 48°C (PBS) or 37°C (PBS plus serum). Liposomes were used within 30 min of separation. The final ET-18-OCH_3 concentration was between 20 and 1000 μM (PBS) or 1 μM (PBS plus serum). The dye efflux was monitored from the increased fluorescence of CF, initially encapsulated within the liposomes at the highly self-quenching concentration of 100 mM. The fluorescence was monitored at 520 nm as a function of time with excitation at 490 nm. 100% release was determined after having added a sufficient amount of Triton X-100 [14]. Fluorescence spectra were recorded with a photon counting spectrofluorometer (Photon Technology International, South Brunswick, NJ) configured for a time based measurement. The fluorescence was normalized as a

Table 1
Hemolytic activity of ET-18-OCH_3 formulations

Name	Composition	Molar ratio	HI_5 (μM) (No. of experiments)	
ET-18-OCH_3	Ether lipid	—	5.6 ± 0.9	(3)
ELL-1	EPC:Chol:ET-18-OCH_3	4:3:3	21	(1)
ELL-2	Chol:DPPG:ET-18-OCH_3	5:3:2	72	(1)
ELL-3	DSPC:Chol:ET-18-OCH_3	4:3:3	20	(1)
ELL-4	EPC:Chol:DPPE-GA:ET-18-OCH_3	1:3:1:5	9	(1)
ELL-5	EPC:Chol:DPPE-GA:ET-18-OCH_3	3:3:1:3	14.1 ± 3	(2)
ELL-6	EPC:Chol:DPPE-GA:ET-18-OCH_3	4:3:1:2	19.0	(1)
ELL-7	EPC:Chol:DPPE-GA:ET-18-OCH_3	5:3:1:1	33	(1)
ELL-8	EPC:Chol:POPE-GA:ET-18-OCH_3	1:3:1:5	14	(1)
ELL-9	EPC:Chol:POPE-GA:ET-18-OCH_3	3:3:1:3	20	(1)
ELL-10	EPC:Chol:POPE-GA:ET-18-OCH_3	4:3:1:2	31.5 ± 2.3	(2)
ELL-11	EPC:Chol:POPE-GA:ET-18-OCH_3	5:3:1:1	31	(1)
ELL-12	DOPC:Chol:DOPE-GA:ET-18-OCH_3	4:3:1:2	250 ± 25	(5)
ELL-13	DSPC:Chol:DPPE-GA:ET-18-OCH_3	1:3:1:5	9.0	(1)
ELL-14	DSPC:Chol:DPPE-GA:ET-18-OCH_3	3:3:1:3	10.5 ± 1.8	(2)
ELL-15	DSPC:Chol:DPPE-GA:ET-18-OCH_3	4:3:1:2	18	(1)
ELL-16	DSPC:Chol:DPPE-GA:ET-18-OCH_3	5:3:1:1	20	(1)
ELL-20	DOPE:Chol:DOPE-GA:ET-18-OCH_3	4:3:1:2	640 ± 60	(2)
ELL-30	EPC:Chol:DOPE-GA:ET-18-OCH_3	4:3:1:2	140	(1)
ELL-40	POPC:Chol:DOPE-GA:ET-18-OCH_3	4:3:1:2	45	(1)
ELL-28	DSPC:Chol:DOPE-GA:ET-18-OCH_3	4:3:1:2	22	(1)

Hemolysis: Lysis of human red blood cells (RBC) by free or liposomal ET-18-OCH_3 was quantitated by hemoglobin release. Various concentrations (3 μM–2 mM) of free or liposomal ET-18-OCH_3, or ET-18-OCH_3 free (plain) liposomes, (1:1 v/v, blood:formulation, serially diluted) were incubated with 4% RBC (washed in PBS, 3 ×) at 37°C for 20 h. Release of hemoglobin from an equal number of RBC by hypotonic lysis in water was taken as 100% positive control; incubation with PBS served as a negative control. After 20 h of incubation, samples were centrifuged at 2000 × *g* for 10 min and the absorbance of the supernatant was measured at 550 nm. Each formulation was tested in triplicate in each experiment. The mean concentrations required to cause 5% hemolysis (HI_5) were calculated by extrapolating between concentrations above and below 5% hemolysis.

percentage of the total fluorescence, which was estimated after disrupting the liposomes with Triton X-100.

2.6. Monolayer studies

The mean molecular area per molecule (MMAM) of ET-18-OCH_3 formulations was determined using a Langmuir–Blodgett mini-trough equipped with a dual barrier mechanism and a Wilhelmy balance (KSV Instruments, Trumbull, Connecticut). ET-18-OCH_3 formulations were prepared from powder and dissolved in a solvent system of hexane:ethanol, 9:1. Mixtures were made to contain an overall lipid concentration of 1.5 mM which was verified by phosphate [15] assays. Chol was determined by HPLC as described above. Monolayers were formed by dropwise addition of sample onto the aqueous surface (10 mM HEPES, 150 mM NaCl buffer, pH 7.2) at the center of the trough. The sample was added until the surface pressure just started to rise above zero, at which time the exact volume of sample added was recorded. After waiting 3 min for solvent evaporation and the monolayer to stabilize, the surface area was reduced at a rate of 150 mm^2/min and surface pressure recorded. The solution level was maintained 1 mm below the top edge of the trough to avoid leakage; solution contact was maintained with the barriers (composed of Delrin) due to their hydrophilic nature. MMAMs were determined by extrapolating the steepest portion of the pressure–area curve to the value at zero surface pressure. For calculation of the expected MMAM (A_0), the values of MMAM (A) for the individual components were added proportionately according to their mole fraction, that is, $A_0 = x_1 \cdot A_1 + x_2 \cdot A_2 + \ldots + x_n \cdot A_n$, where x is the mole fraction of each component. All experiments were done at 23°C.

2.7. Association of ET-18-OCH_3 with liposomes

Continuous sucrose gradients (from 2 to 20% and from 5 to 15% sucrose in water) were made using a Gradient Master (BioComp, Fredericton, NB, Canada) at 15 rpm speed and 81.5°C, using Beckman Ultra-Clear centrifuge tubes (14×89 mm). 0.5 ml from various ET-18-OCH_3-liposomal formulations in PBS were added at the top of the tube and centrifuged (20 h) at 200,000 g (5°C). Fifty 250 μl fractions were collected, starting from the top of the tubes, followed by analysis by reverse-phase HPLC, using the method previously described.

3. Results

3.1. Rationale for liposome compositions investigated

The overall aim of this work was to identify ET-18-OCH_3 liposomes suitable for clinical investigation by the intravenous route.

Cholesterol is known to generally enhance the in vivo stability of liposomes [16,17] and was recently shown to pack favorably with ET-18-OCH_3 in a 1:1.5 mole ratio [10]. Thus ET-18-OCH_3:cholesterol assemblies were formulated with saturated and unsaturated lipids in order to assess the overall stability of multicomponent systems in plasma. The role of the phospholipid head group was investigated as the geometry and packing of lipids is known to play an important role in liposome supramolecular assembly [18]. Most formulations included a negatively charged component to decrease aggregation [19]. In addition, the inclusion of glutaric acid derivatized phospholipids (double negative charge) was investigated because these derivatives have been shown to enhance liposome circulation time [20].

As the maximum incorporation of ET-18-OCH_3 concomitant with the maintenance of other desirable properties was of obvious importance, the effects of variations in the mol% of ET-18-OCH_3 in otherwise similar liposomes were also compared.

In initial experiments it was observed, at the light microscope level, that ET-18-OCH_3 readily formed extrudable non-aggregated liposomes with various different liposome components. These liposomes were used in the hemolysis experiments (Table 1).

3.2. Hemolysis

The liposome compositions investigated and the results of hemolysis experiments are shown in Table 1. The HI_5 was used as a comparative measure of minimal hemolytic activity. Free ET-18-OCH_3 had an HI_5 of 5–6 μM, less than the HI_5 for all of the liposome formulations tested. Uncharged formula-

Table 2
Characteristics of some ET-18-OCH_3-liposome preparations

Name	% Drug recovery (No. of experiments)	Mean diameter ± S.D. (nm)	Captured volume (μl/μmol)
ELL-20	100 ± 15 (3)	98 ± 19	n.d.
ELL-12	100 ± 15 (4)	87 ± 24	0.71
ELL-40	95 ± 5 (2)	95 ± 28	0.89
ELL-28	85 ± 3 (2)	159 ± 65	0.31
ET-18-OCH_3	n.a.	11 ± 1	n.a.

Drug recovery (% drug retained during liposome preparation), mean diameter and capture volume of various ET-18-OCH_3 liposomal formulations. The chemical composition of the formulations was X:Chol:DOPE-GA:ET-18-OCH_3 = 4:3:1:2 mol, where X is DOPE for ELL-20, DOPC for ELL-12, POPC for ELL-28 and DSPC for ELL-28.
n.d. = not determined.
n.a. = not applicable.

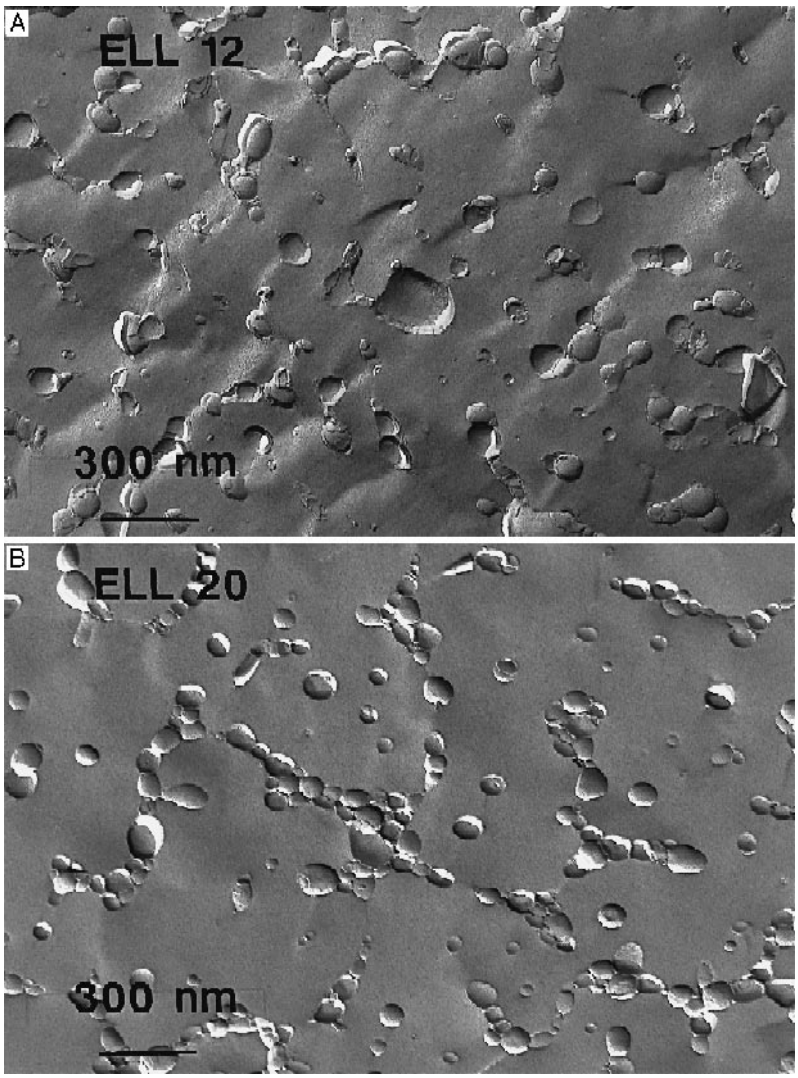

Fig. 1. Freeze fracture electron micrographs of ELL-12 (A) and ELL-20 (B), where the chemical compositions were X:Chol:DOPE-GA:ET-18-OCH_3 = 4:3:1:2 mol and X was DOPC for ELL-12 and DOPE for ELL-20. The bar represents 300 nm.

tions (ELL-1 and -3) attenuated hemolysis about three fold compared to free ET-18-OCH_3. When the series ELL-4–7, 8–11 and 13–16 were inspected it was seen that decreasing the mol% ET-18-OCH_3 increased the attenuation of hemolysis. For series 8–11 and 13–16 the attenuation of hemolysis was similar between 10 and 20 mol% ET-18-OCH_3. The maximum attenuation of hemolysis was somewhat less for a negatively charged saturated phospholipid series (13–16) than for the two unsaturated negatively charged phospholipid series (4–7 and 8–11).

ELL-12 in which EPC was replaced with DOPC, was less hemolytic than any of the EPC formulations. Liposomes prepared using 20 mol% dioleoylphosphatidylethanolamine (DOPE) instead of DOPC (ELL-20) were even less hemolytic than ELL-12. Other formulations were inferior to ELL-12 or ELL-20 with respect to attenuation of hemolysis, including ELL-2 which was composed of 50 mol% Chol plus DPPG as a negatively charged component. The results suggest that both the nature of the phospholipid head group and the overall combined molecular shape of the multi-component system is important for efficient attenuation of hemolysis.

3.3. ET-18-OCH_3-liposome avidity

Some ET-18-OCH_3 liposome preparations were characterized by different methods to determine the affinity of ET-18-OCH_3 to form liposomes with various lipids. A summary of the results is shown in Table 2. For all formulations, with the exception of ELL-28, no significant ET-18-OCH_3 loss occurred during preparation and extrusion. After extrusion the size distributions of the various preparations were Gaussian, consistent with the formation of a single population of liposomes, although ELL-28 was again an exception. All formulations evaluated had captured volumes ranging from 0.31 to 1.0 μl/μmol. For ELL-12 there was no significant change in the chemical composition or liposomal size distribution over a 3 month period.

Fig. 1 shows freeze fracture electron micrographs

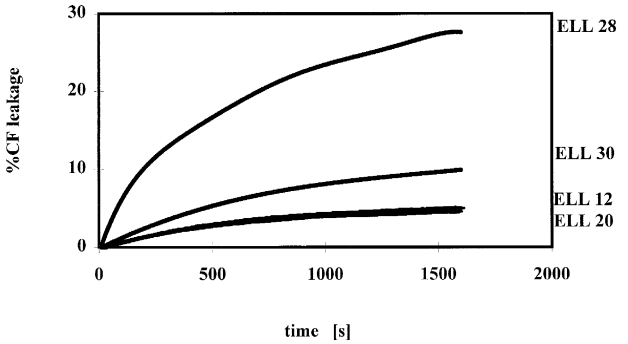

Fig. 2. Time course of carboxyfluorescein (CF) release from ET-18-OCH_3 liposomal formulations in PBS at 48°C. The chemical compositions of the formulations were X:Chol:DOPE-GA:ET-18-OCH_3 = 4:3:1:2 mol, where X is DOPC for ELL-12, DOPE for ELL-20, EPC for ELL-30 and DSPC for ELL-28. The liposomes were diluted 100 fold into 48°C PBS at the beginning of each experiment. The dye efflux was monitored as a function of time, by the increased fluorescence of CF, initially encapsulated within the liposomes at the highly self-quenching concentration of 100 mM. Excitation was and 490 nm and fluorescence was detected at 520 nm. Leakage is expressed as an increase in CF fluorescence relative to the total CF present after disrupting the liposomes with Triton X-100.

Table 3
Stability of ET-18-OCH_3 liposomal formulations in 0.5% serum at 37°C

Name	Composition	$t_{\frac{1}{2}}$
	(mol%)	(min)
ELL-20	DOPE:CHOL:DOPE-GA:ET-18-OCH_3, 4:3:1:2	1080
ELL-12	DOPC:CHOL:DOPE-GA:ET-18-OCH_3, 4:3:1:2	260
ELL-30	EPC:CHOL:DOPE-GA:ET-18-OCH_3, 4:3:1:2	4.7
ELL-40	POPC:CHOL:DOPE-GA:ET-18-OCH_3, 4:3:1:2	0.42
ELL-28	DSPC:CHOL:DOPE-GA:ET-18-OCH_3, 4:3:1:2	0.13

Comparison of the $t_{\frac{1}{2}}$ of ET-18-OCH_3 liposomal formulations in the presence of 0.5% serum. Liposomes were diluted 500 fold into preheated 37°C PBS, containing 0.5% serum at the beginning of each experiment. Dye efflux was monitored by increase of fluorescence at 520 nm using excitation wavelength 490 nm and the time required for 50% of carboxyfluorescein entrapped in the liposomes to leak to the external environment was calculated ($t_{\frac{1}{2}}$). 100% release was determined after having added sufficient amount of Triton X-100 resulting in disruption of liposomes and release of entrapped dye.

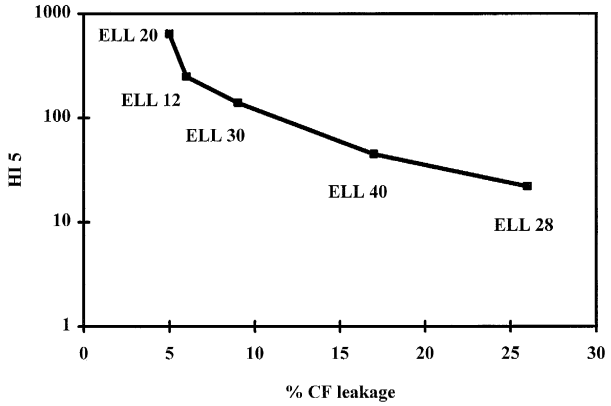

Fig. 3. Comparison of the hemolytic activity (Table 1) and carboxyfluorescein leakage (Fig. 2) in ET-18-OCH_3 liposomes. The CF release is expressed as a percentage of the total after 25 min incubation with PBS at 48°C.

of ELL-12 (Fig. 1a) and ELL-20 (Fig. 1b) preparations. In both cases typical spherical liposome structures of relatively uniform size, around 100 nm, can be seen. Most of the liposomes were unilamellar with some multilamellar structures visible. There was little visible aggregation in ELL-12 although it was possible there was more aggregation in ELL-20.

3.4. Carboxyfluorescein permeability

CF has been widely used as a sensitive indicator for liposome permeability [21] and Fig. 2 shows there was a time dependent permeation of CF from ET-18-OCH_3 liposomes in buffer at 48°C. This temperature was used because permeation at 37°C was slow. It can be seen that the DOPC and DOPE formulations (ELL-12 and ELL-20) were the least permeable of the formulations tested, with less than 6% CF leaking out in 25 min compared to over 27% for the DSPC containing ELL-28 formulation. Table 3 shows that ELL-20, followed by ELL-12, released CF at the slowest rates in 0.5% serum. Fig. 3 is a plot of CF leakage versus hemolytic activity in buffer. Formulations with slower CF release rates were less hemolytic, possibly reflecting tighter lipid packing.

Table 4
Mean molecular area per molecule (MMAM) for monolayers of ELL lipid mixtures with or without ET-18-OCH_3 present

MMAM (Å[b]/molecule)			
Formulation [a]	Expected	Actual	Difference
ELL-12	72.0	51.3	−20.7
ELL-12 without ET-18-OCH_3	70.0	60.3	−9.7
ELL-20	71.8	53.3	−18.5
ELL-20 without ET-18-OCH_3	70.2	58.7	−11.5
ELL-40	71.9	49.3	−22.6
ELL-40 without ET-18-OCH_3	69.5	57.7	−11.8

[a] Lipid molar ratios for formulations without ET-18-OCH_3 were 4:3:1 phospholipid/Chol/DOPE-GA.
[b] Expected MMAM is the value calculated for the mixtures based on simple additivity of the MMAM for individual components. Actual MMAM were those values determined experimentally as outlined in Section 2.

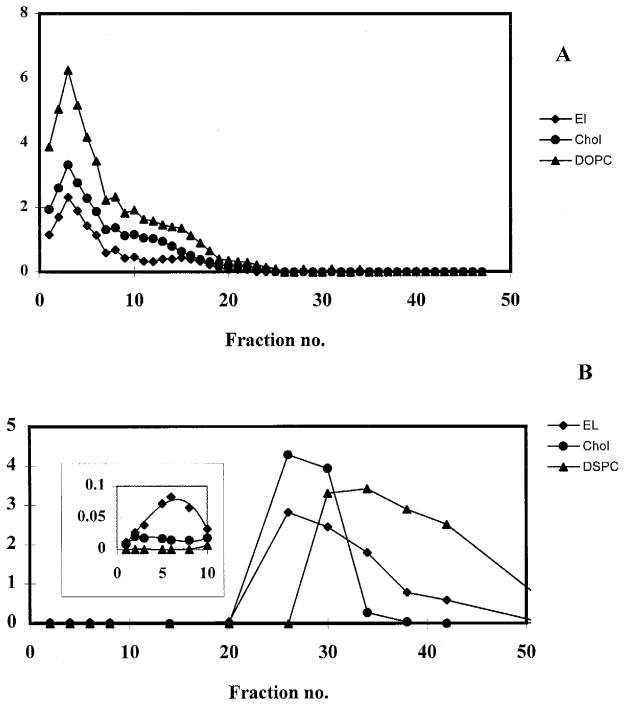

Fig. 4. Homogeneity of DOPC and DSPC based ET-18-OCH_3 liposomal formulations. (ET-18-OCH_3), Chol and DOPC (A) of DSPC (for 4B) concentrations present in fractions collected after density gradient centrifugation of ET-18-OCH_3 liposomal formulations. The chemical compositions of the formulations were X:Chol:DOPE-GA:ET-18-OCH_3 = 4:3:1:2 mol, where X is DOPC (A) and DSPC (B). The gradient was 2 to 20% sucrose (A) and from 5 to 15% sucrose (B). Formulations were centrifuged for 20 h at 200,000× g at 5°C. Fifty fractions were collected and analyzed by reverse phase HPLC (Inertsil ODS2 150×4.6 mm column) with an evaporative light scattering detector using 4% water in methanol as a mobile phase. The insert in (B) is an enlargement of the 0–10 fraction section on the main chart. The symbols used are the same as in the main chart.

3.5. Monolayer studies

As complementary packing between lipids facilitates the formation of lamellar structures [18,22] it was thought possible that complementary packing between ET-18-OCH_3 and other lipids could play a role in reducing the transfer of ET-18-OCH_3 from liposomes to red cell membranes. To assess the degree of shape complementarity for the formulations used here, we measured the mean molecular area per molecule (MMAM) for monolayers of lipid mixtures spread upon a Langmuir trough. Table 4 shows the MMAM of the lipid components of three ether lipid liposome formulations with (four component) and without (three component) the inclusion of ET-18-OCH_3. It can be seen that MMAM values with ET-18-OCH_3 present were significantly less (19–23 $Å^2$ per molecule) than those predicted based on simple additivity of the individual components (see Section 2 for details). These reductions in MMAM were consistent with a significant degree of shape complementarity [22].

3.6. Homogeneity of ET-18-OCH_3 association with liposomes

ET-18-OCH_3 liposome formulations with large differences in the HI 5 value were characterized for homogeneity using density gradient centrifugation. Fig. 4 shows that most of the lipids from the DOPC formulation (ELL12) were found towards the top of the gradient (fractions 1–15) whereas lipids from the DSPC formulation (ELL-28) were found in fractions 20 and higher. It can also be seen in Fig. 5A that the values for the individual components paralleled one another for the DOPC formulation whereas they did not for the DSPC formulation. Fig. 5B shows that the DOPC/ET-18-OCH_3 and Chol/ET-18-OCH_3 mole ratios remained constant in all fractions in ELL-12 whereas the Chol/ET-18-OCH_3 ratio was increased in fractions 24–30 and DSPC/ET-18-OCH_3 was increased in fractions 35–40 for ELL-28. The results indicate that ELL-12 is composed of a much more compositionally homogeneous population of liposomes compared to ELL-28. In another experiment, we found that ELL-20, another non-hemolytic formulation, was as homogenous as ELL-12.

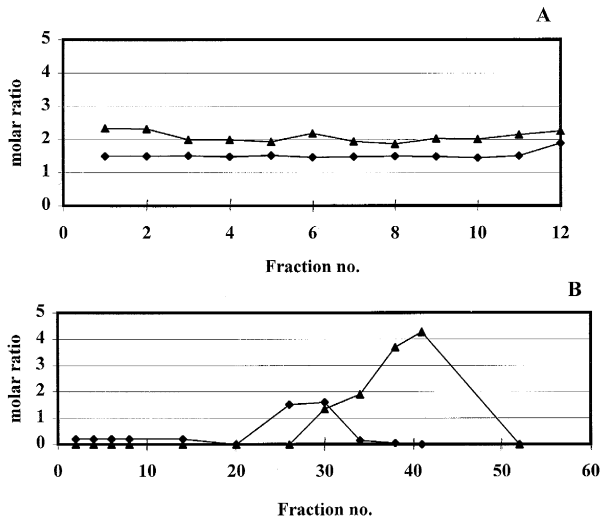

Fig. 5. Calculated Chol/ET-18-OCH_3 (A and B, solid triangles) and DOPC/ET-18-OCH_3 (A) or DSPC/ET-18-OCH_3 (B) molar ratios of fractions collected after density gradient centrifugation of DOPC (A) or DSPC (B) based ET-18-OCH_3 liposomal formulations (see legend to Fig. 4).

4. Discussion

The results show that association of ET-18-OCH_3 with liposomes attenuated hemolysis in a composition dependent manner.

We used a hemolysis assay to compare different liposome formulations because we believed hemolysis reduction by liposome entrapment to be a useful indicator of liposome stability. ET-18-OCH_3 forms micelles in water above its critical micellar concentration, which is approximately 0.5 μM [23], and hemolysis occurs at concentrations above this value probably as a result of critical accumulations in RBC membranes. The attenuation of hemolysis may be due to a decrease in the amount of solution phase ET-18-OCH_3, reflecting its stable association with liposome membranes. However, we cannot rule out that hemolysis could take place as a result of the transfer of ET-18-OCH_3 directly to RBC through processes mediated by liposome-RBC collision.

A much greater attenuation of hemolysis occurred using DOPC or DOPE (di-C18, unsaturated fatty acid chains) as the major neutral phospholipid components, rather than DSPC (di-C18, saturated fatty acid chains). However EPC (mixed C16 and C18, saturated and unsaturated fatty acid chains) attenuated hemolysis at least as well as DSPC suggesting that

fatty acid chain saturation is not the only factor involved in hemolysis attenuation. Hemolysis attenuation was also dependent on negatively charged phospholipid components in otherwise similar liposomes. DOPE-GA (di-C18) attenuated hemolysis more than POPE-GA (C16, C18) or DPPE-GA (di-C16) in EPC liposomes suggesting that matching neutral or negatively charged C18 phospholipid fatty acid chains form the most stable, least hemolytic, liposomes in association with ET-18-OCH_3, which itself has a single C18 fatty acid chain. The presence of DOPE attenuated hemolysis more than DOPC. This suggests that the phospholipid head group and the overall macromolecular structure assembly plays an important role in stable liposome architecture. The Langmuir trough data strongly supported the hypothesis that in two component systems the more cone-like DOPE packs better with the inverted cone-like ET-18-OCH_3, than with the more cylindrical DOPC [22]. The data presented in this report also show that when ET-18-OCH_3 was included in multi-component DOPE or DOPC liposomes, the mean molecular area decreased more than would be expected. Thus, lipids pack tighter in ET-18-OCH_3 liposomes. The rate of CF permeation from ET-18-OCH_3 liposomes may also be related to the packing of lipid molecules in the lipid bilayers because CF (a charged water soluble compound) cannot permeate easily through well packed lipid bilayers when it is entrapped in the aqueous space of liposomes [21].

Alkyl phospholipid derivatives have been investigated previously in order to elucidate their physico–chemical properties [24,25] and to elucidate their interactions with model membranes including phospholipids and liposomes [26–28]. In addition, ether lipids other than ET-18-OCH_3 have been entrapped in liposomes and investigated for their biological properties [29,30]. For example, ET-18-OCH_3 was entrapped in PC:Chol liposomes to a maximum of 10 mol% and it was shown to inhibit ^{3}H-thymidine incorporation in L1210 and L929 cells in vitro [9]. In a subsequent report [31], the same group, using the same type of liposome, reported that ET-18-OCH_3 liposomes could activate macrophages. However, no systematic exploration of the effects of modification of liposome composition in in vitro biological systems has been reported, nor have any in vivo studies accomplished. Some investigations of the role of liposome composition, including inclusion of negatively charged lipids, on in vitro cytotoxicity and anti-tumor effects were accomplished using other ether lipids [9], but again no systematic investigations have been described. Recent reports [30,32] describe results using hexadecylphosphocholine (Miltefosine) entrapped in small unilamellar vesicles composed of dicetylphosphate:Chol:HPC in a 1:10:10 molar ratio. Reduced hemolysis by liposome formulations was observed.

Although no direct extrapolation can be made between the studies reported herein and in vivo effects of ET-18-OCH_3, it is noteworthy that in animal and clinical studies hemolysis is a significant toxicity and may be dose limiting in some circumstances [2,3]. It is therefore possible that liposome formulations of ET-18-OCH_3 which have been shown to have greatly attenuated hemolysis in vitro may also be less hemolytic in vivo. Formulations where phospholipids pack efficiently with ET-18-OCH_3, such as ELL-12 or 20, which are relatively non-hemolytic and non-permeable in buffer and serum, are therefore prime candidates for investigation as in vivo delivery systems for ET-18-OCH_3 [33].

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