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Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration

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Abstract

Liposomes composed of egg phosphatidylcholine (EPC) (13.4% of the acyl chains being polyunsaturated fatty acids (PUFA)) and EPC/cholesterol (10:1 mol/mol) were studied for factors that affect liposomal lipid oxidative damage and hydrolysis upon long-term (16 months) storage. Factors studied include: (1) levels of lipid/water interface hydration, related to the presence of cholesterol in the lipid bilayer; (2) the membrane-associated antioxidant vitamin E; (3) the water-soluble antioxidant Tempol; and (4) exposure to light. Liposomal dispersions were stored at room temperature, either exposed to or protected from daylight, for a period of 16 months. Chemical and physical changes were monitored at several time points to assess oxidative and hydrolytic degradation of liposomal lipids. The conclusions of the study are: (1) PUFA are the most sensitive component of the liposome bilayer to oxidative degradation damage during long-term storage; (2) EPC liposomes are more sensitive to degradation during storage than EPC/cholesterol liposomes, the presence of cholesterol in the lipid bilayer having a protective effect, probably due to its effect in decreasing the lipid-bilayer hydration; (3) oxidative degradation is the major process during long-term storage, having an earlier onset than the hydrolytic degradation; and (4) Tempol provided significantly better protection than vitamin E to EPC liposomal PUFA against oxidative damage during long-term storage. The relevance of cholesterol's presence, as a 'drying agent', in membranes containing PUFA to resistance of biological membranes to oxidative damage is discussed.

Keywords: Liposomes; Stability; Tempol; Nitroxides; Lipid peroxidation; Vitamin E

1. Introduction

The assembly of lipids in a membranal structure imparts protection against oxidative damage and hydrolysis (Barclay and Ingold, 1981). The various factors that contribute to this protection are related to the organization of lipids in the membrane assembly. Liposomes, in which compo-

Abbreviations: EPC, egg phosphatidylcholine; EPR, electron paramagnetic resonance; GC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; LPO, lipid peroxidation; LUV, large unilamellar vesicles; NEFA, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; SUV, small unilamellar vesicles; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

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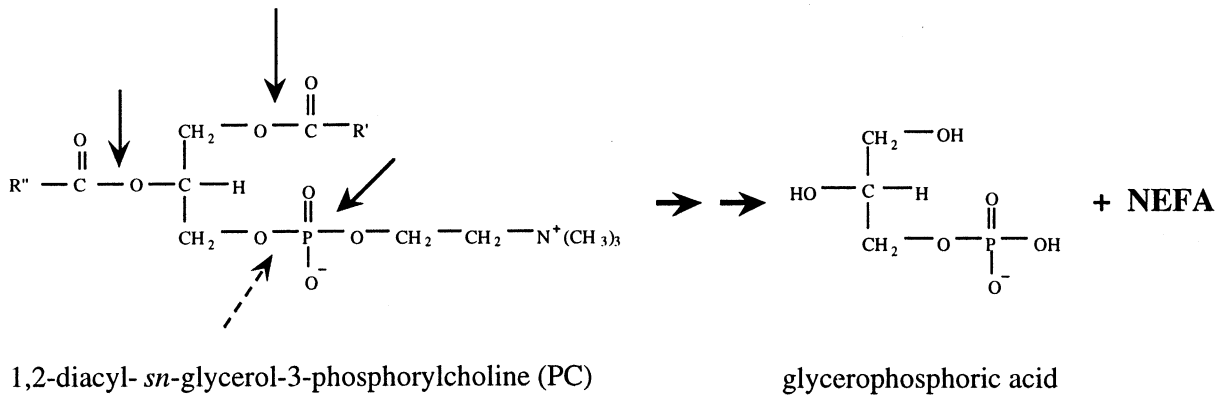

Scheme 1. PC and hydrolysis end products, glycerophosphoric acid + NEFA.

sition, structure and dynamics can be fully controlled (Lichtenberg and Barenholz, 1988; Barenholz and Crommelin, 1994), are an excellent system to study why and to what extent such factors are involved. Liposomes are also an important system in their own right in medical, cosmetic, and industrial applications (Lasic, 1993; Barenholz and Lasic, 1996; Lasic and Barenholz, 1996). Their instability upon storage is one of the major obstacles to their use (Lasic, 1993). Liposome stability involves physical, chemical, and biological stability of the particle itself and its constituents. Phospholipids (in particular, phosphatidylcholine) and cholesterol, which are the main components of biological membranes, are very common components of liposomes used for drug delivery both in approved drugs and clinical trials (Gabizon et al., 1991; Adler-Moore and Profitt, 1993; Gabizon et al., 1994; Lasic and Papahadjopoulos, 1998).

The presence of cholesterol in pharmaceutical liposomal formulations has several known beneficial effects, such as increased retention of water-soluble drugs during storage, prevention of lipid phase transition above 0°C, and increased resistance of liposomes to in vivo degradation (Grit and Crommelin, 1992; Gabizon et al., 1998). Cholesterol has been suggested to also play a role as antioxidant in biological membranes (Smith, 1991). For phospholipids in aqueous liposomal dispersions, two degradation pathways have been described that might limit shelf-life: the oxidative and hydrolytic degradation pathways. Oxidation of phospholipids in liposomes involves mainly those with unsaturated fatty acyl chains, and operates via a free radical chain mechanism. The hydrolytic pathway involves all four ester bonds present in the glycerophospholipid molecule (Scheme 1) (especially the two acyl esters), where lysophospholipids are intermediate hydrolysis products, and non-esterified fatty acids (NEFA) and glycerophospho compounds are the end products (Grit and Crommelin, 1993; Shmeeda et al., 2000). The hydrolysis of the ester bond between glycerol and phosphoric acid (dashed arrow in Scheme 1) appears to be very slow and, therefore, no free phosphoric acid and glycerol are produced (Grit et al., 1993).

Minimizing lipid peroxidation includes the use of high-quality raw materials (purified by removing hydroperoxides and transition metal ions), storage at low temperatures, and protection from daylight. Other means of protection include use of antioxidants such as α-tocopherol or butylated hydroxytoluene and chelating agents such as ethylenediamine tetraacetic acid, diethylenetriamine pentaacetic acid (Niki, 1987), or desferal (Amselem et al., 1992; Barenholz and Amselem, 1993). Keeping the surface pH in the range 6.3–7.0, which may be different than the bulk pH, is crucial for minimizing phospholipid hydrolysis (Lichtenberg and Barenholz, 1988; Grit et al., 1993; Zuidam and Barenholz, 1997).

We aimed to study factors influencing chemical and physical stability of membrane lipids in liposomes during long-term storage of egg phosphatidylcholine (EPC) and EPC/cholesterol (10:1) liposomes, focusing on the effect of: (1) cholesterol's presence in the liposome bilayer; and (2) antioxidants added to the liposome dispersions during preparation and storage. EPC was selected mainly due to its acyl chain composition, which resembles many biological membranes. We compared the antioxidant activity of the naturally occurring, membrane-residing, vitamin E with that of the water-soluble 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), a stable cyclic nitroxide radical, which has previously been shown to efficiently inhibit radiation-induced lipid degradation. (Samuni and Barenholz, 1997; Samuni et al., 1997) Liposomal dispersions were stored at room temperature, either exposed to or protected from daylight. Liposomal lipid degradation was studied using gas–liquid chromatography (GC) and high-performance liquid chromatography (HPLC) analyses after 3, 6, and 10 months of storage. The results show that cholesterol, vitamin E, and Tempol protect the EPC polyunsaturated fatty acids (PUFA) against oxidative damage, and that the hydrolytic degradation is much slower than the oxidative damage. Both types of damage are affected by the hydration at the lipid/water interface.

2. Materials and methods

2.1. Materials

N-2-Hydroxyethylepiperazine-*N*′-2-ethanesulfonic acid (Hepes), cholesterol 99 + %, and D-α-tocopherol were purchased from Sigma (St. Louis, MO, USA); 1-α-phosphatidylcholine (egg) (> 99%) from Avanti Polar Lipids (Alabaster, AL, USA); sodium hydroxide from Frutarom (Haifa, Israel); Tempol from Aldrich (Milwaukee, WI, USA); Meth-Prep II kit from Alltech Associates (Deerfield, IL, USA); Triton X-100 from Packard Instrument (Downers Grove, IL, USA); hexane and isopropanol (HPLC grade) from BioLab (Jerusalem, Israel); 6 - dodecanoyl - 2 - dimethylaminonaphthalene (Laurdan) from Lambda (Graz, Austria); and *N*,*N*-dimethylformamide from BDH (Poole, UK). All chemicals were of analytical grade. Water was doubly distilled and deionized.

2.2. Liposome preparation

EPC small unilamellar vesicles (SUV)[1] of unimodal size distribution of 60 ± 30 nm in 10 mM Hepes buffer (with 0.13 M NaCl) (pH 7.4) were prepared by extrusion using the LiposoFast-Basic device (Avestin, Ottawa, Ont., Canada) (MacDonald et al., 1991). The final phospholipid concentration was 20 mM.

EPC/cholesterol (10:1) (mol/mol) SUV liposomes were prepared by dissolving the two lipids in tertiary butanol, lyophilization of the lipid mixture, resuspension in 50 mM Hepes buffer (pH 7.4) and extrusion using the LiposoFast-Basic device. The final phospholipid concentration was 17 mM, and the final cholesterol concentration was 1.5 mM. There was a monomodal size distribution of 80 ± 20 nm.

2.3. Determination of phospholipid concentration

Phospholipid concentration was determined using a modification of the Bartlett procedure (Barenholz and Amselem, 1993).

2.4. Particle size determination

Liposome size distribution was determined by photon correlation spectroscopy using a Coulter Model N4 SD apparatus (Barenholz and Amselem, 1993).

2.5. Acyl chain oxidation

Oxidative damage to each of the individual EPC acyl chains was determined by following changes in acyl chain composition using GC anal-

[1] SUV are liposomes that show significant differences in packing between their external and internal monolayers (Lichtenberg and Barenholz, 1988). The cut-off between SUV and large unilamellar vesicles (LUV) is at ~100 nm diameter.

ysis as described previously by Barenholz and Amselem (1993). In brief, after a Bligh and Dyer extraction, (Bligh and Dyer, 1959) the lower (chloroform-rich) phase was transferred to a small glass bottle, evaporated under N_2 to complete dryness, and dissolved in 50 μl toluene. Transmethylation was performed by 30 min of incubation at room temperature in the presence of 20 μl Meth-Prep II (Alltech). A volume of 2 μl of this mixture was injected into a Perkin Elmer AutoSystem GC and Autosampler, using a 6-foot 10% Silar 10C column (Alltech), dry N_2 as the carrier gas, the flame ionization detection. The initial temperature of the run was 140°C for 5 min, then the oven temperature was raised at a rate of 5°C min^{-1} up to 240°C, and then kept there for 5 min. Methyl esters were identified by comparing their retention times with those of known standards. Methyl palmitate (C_{16}), a saturated acyl chain, which had been previously found to be highly resistant to peroxidation by γ-irradiation (Zuidam et al., 1996; Samuni and Barenholz, 1997) (confirmed in the present study using methyl pentadecanoate (C_{15}) as an external standard), was selected as an internal reference for the determination of the extent of degradation in other acyl chains of EPC.

2.6. Determination of phospholipid hydrolysis: by quantification of NEFA

Non-esterified (or free) fatty acid (NEFA) concentration in the samples was determined (as a means to quantify phospholipid hydrolysis) using the NEFA C Kit by Wako (Wako Chemicals GmbH, Neuss, Germany). The details are described elsewhere (Shmeeda et al., 2000). In brief, samples were diluted to adjust the kit's sensitivity range, and 60 μl aliquots of the diluted dispersion were drawn into Nunclon microwell plates. Twenty microliters of 20% Triton X-100 in water (fresh solution) was added to all samples to solubilize the liposomal dispersion. Following addition of color reagents A and B, and the proper incubation times at 37°C, the optical density of the samples at 540 nm was measured using a Biochromatic ELISA Reader (Labsystems Multiskan, Finland). NEFA concentration in the samples was calculated using the kit standard curve.

2.7. Quantification of cholesterol and α-tocopherol

The amount of cholesterol and α-tocopherol degradation was quantified from reduction in cholesterol and α-tocopherol level, and appearance of cholesterol's major degradation product 7-keto-cholesterol, following extraction of the aqueous liposomal dispersion, by HPLC using the procedure of Ansari and Smith (1979). Extraction was done using the Dole extraction procedure (Barenholz and Amselem, 1993) where the heptane-enriched upper phase, containing $>98\%$ of the cholesterol and 7-keto-cholesterol, was concentrated and analyzed. The analysis was performed at ambient temperature on an Econosphere silica column (10×0.46 mm, I.D.) using a silica pre-column (Alltech). The mobile phase consisted of hexane:isopropanol 500:6 (v/v) at a flow rate of 1 ml min^{-1}. The system consisted of a Kontron (Switzerland) HPLC system, 425 pump, 430 detector, 460 automatic injector, and 450 data analysis system. Spectrophotometric detection was carried out at 212 nm.

2.8. Quantification of Tempol

Electron paramagnetic resonance (EPR) spectrometry, using a JES-RE3X ESR spectrometer (JEOL, Japan), was employed to quantify changes in Tempol concentrations. Samples were drawn by a micropippette into a gas-permeable Teflon capillary of 0.81 mm I.D., 0.05 mm wall thickness, and 15 cm length (Zeus Industrial Products, Raritan, NJ, USA). Each capillary was folded twice, inserted into a 2.5 mm I.D. quartz tube open at both ends, and placed in the EPR cavity. EPR spectra were recorded with the center field set at 3361 G, 100 kHz modulation frequency, 1 G modulation amplitude, and non-saturating microwave power.

One of the major mechanisms by which nitroxides ($>$N–O) decay in biological systems is through a one-electron reduction process yielding hydroxylamines ($>$N–OH) (Swartz et al., 1986). For determination of the total concentration of nitroxide + hydroxylamine, ferricyanide to a final concentration of 1 mM (another alternative is using H_2O_2 + NaOH, final concentrations 0.3%

and 0.01 N, respectively) was added to the sample to oxidize the hydroxylamine, Tempol-H, to the nitroxide, Tempol.

2.9. Experimental conditions

All samples were prepared in triplicate in clear-glass vials with plastic screw caps. The samples were with or without antioxidants. For EPC liposomes, the following dispersions were prepared: (1) EPC liposomes alone; (2) EPC liposomes + 1 mM Tempol; and (3) EPC liposomes + 1 mM vitamin E. For EPC/cholesterol liposomes, the following dispersions were prepared: (1) EPC/cholesterol liposomes alone; (2) EPC/cholesterol liposomes + 0.75 mM Tempol; (3) EPC liposomes + 1 mM vitamin E; and (4) EPC/cholesterol liposomes + 0.1 mM vitamin E.

Previous results demonstrated a bell-shaped dose–response curve for the protective activity of vitamin E (O. Tirosh, unpublished results, 1997). The concentrations of vitamin E which provided optimal protection ranged from 0.1 to 1 mol%, whereas below and above these values, vitamin E was significantly less effective. Therefore, 0.1 mM (0.5 mol%) of vitamin E has been selected. The effect of 1 mM vitamin E (5 mol%) has also been tested to compare it with that of a comparable concentration of Tempol. All samples were placed on a shelf at room temperature. One-half of the samples were wrapped with aluminum foil. The rest were exposed to room daylight in vials of glass that absorb the UV radiation. The follow-up of the effect on the two different lipid compositions was carried out for 16 months.

2.10. Cholesterol-induced bilayer hydration changes: expressed by the generalized fluorescence polarization of the fluorophore Laurdan

The fluorescence properties of Laurdan are notably sensitive to the polarity of the environment (Parasassi et al., 1991). Laurdan has been used extensively to follow changes in hydration level of lipid vesicles (reviewed by Parasassi and Gratton (1995)). The changes in Laurdan's fluorescence properties are defined by a steady-state fluorescence parameter called generalized polarization (GP).

The excitation GP value was calculated as:

$$\text{Excitation } GP_{340} = (I_{440} - I_{490})/(I_{440} + I_{490})$$

where I_{440} and I_{490} are the intensities of the emission at wavelengths 440 and 490 nm (Parasassi and Gratton, 1995) at an excitation wavelength of 340 nm.

The emission GP value was calculated as:

$$\text{Excitation } GP_{440} = (I_{410} - I_{340})/(I_{410} + I_{340})$$

where I_{340} and I_{410} are the intensities of the excitation at wavelengths 340 and 410 nm at the emission wavelength of 440 nm.

Laurdan-labeled EPC/cholesterol LUV were prepared by mixing tertiary butanol solutions of the different lipids with Laurdan in *N*,*N*-dimethylformamide at a ratio of 1 mole of fluorophore per 1000 moles of lipids. Following lyophilization of the lipid mixture and resuspension in 20 mM Hepes buffer (pH 7.4), LUV were prepared by extrusion using the LiposoFast-Basic device, as already described. Final phospholipid concentration was 40 mM, with varying cholesterol concentration of 0, 10, 20, 30, 35, 40, and 45 mol%.

2.10.1. Fluorescence measurements

An aliquot of Laurdan-labeled liposomes was diluted with 3 ml of 20 mM Hepes buffer (pH 7.4) to the desired concentration. Fluorescence measurements at the already specified wavelength were performed on a Perkin Elmer LS50B luminescence spectrometer using a 1 cm light path cell.

3. Results

3.1. Liposome physical and chemical stability

The effect of liposome long-term storage at room temperature, in the dark or exposed to daylight, was studied by measuring liposome size and pH, and quantifying cholesterol degradation using HPLC, phospholipid hydrolysis by NEFA levels, and acyl chain oxidation by analyzing phospholipid acyl chain composition using GC.

3.2. Liposome size

3.2.1. EPC liposomes

After extrusion, the average size of extruded liposomes was 60 ± 30 nm. After 12 months of storage, the EPC liposomes without antioxidants showed an increase in liposome size. Size distribution shifted to 120 ± 14 nm, while size change of the liposomal dispersion containing vitamin E was minimal (69 ± 20 nm), and for the dispersions containing Tempol, no significant change in size occurred.

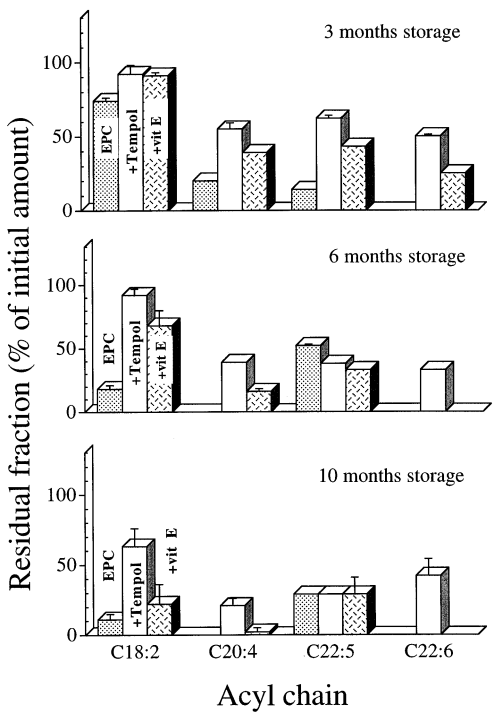

Fig. 1. Effect of Tempol and vitamin E on damage caused to acyl chain residues of EPC upon long-term storage, exposed to daylight. Liposomal dispersions of EPC SUVs in 10 mM Hepes buffer (pH 7.4) were stored at room temperature for a period of 10 months, with and without 1 mM Tempol or vitamin E. Acyl chain composition was determined after methyl esterification followed by GC separation. The levels of acyl chains at 3, 6 and 10 months of storage, related to palmitic acid, which served as internal standard, are presented as a fraction of the original levels.

3.2.2. EPC/cholesterol (10:1) liposomes

After extrusion, a fresh SUV preparation has an average size of 80 ± 20 nm. After 6 months of storage, no significant change in size was measured in any of the liposomal dispersions.

3.3. pH changes

3.3.1. EPC liposomes

Three months after preparation, all samples (pH 7.4 at preparation) showed a decrease in pH of 0.5–0.8, with no difference associated with antioxidant content. The pH measurements after 16 months showed an average pH value of 5.1 ± 0.1 for EPC liposomes, 5.6 ± 0.7 for the dispersions containing vitamin E, and 6.2 ± 0.4 for the dispersions containing Tempol.

3.3.2. EPC/cholesterol (10:1) liposomes

No significant change in pH (ΔpH 0.0–0.3) was found in any of the liposome dispersions, with or without antioxidants (pH 7.4 at preparation), after 3 and 6 months of storage. After 16 months of storage, all samples showed a decrease in pH to 7.0 ± 0.08.

3.4. Acyl chain peroxidation

3.4.1. EPC liposomes

Degradation of the acyl chain residues of the phospholipids following 3, 6, and 10 months of storage was measured using GC, which enables an accurate quantification of each of the acyl chains. The results of acyl chain analysis of the liposomal dispersions exposed to daylight are summarized in Fig. 1. The dispersions that were not exposed to daylight showed similar results (namely, there was no effect of daylight). As can be seen from the results, the more sensitive PUFA, such as arachidonic acid ($C_{20:4}$), *cis*-7,10,13,16,19-docosapentaenoic acid ($C_{22:5}$), and *cis*-4,7,10,13,16,19-docosahexaenoic acid ($C_{22:6}$) mostly or completely disappeared after 6 months of storage. Such PUFA were partially preserved in the presence of 1 mM Tempol, which was significantly more effective than 1 mM vitamin E.

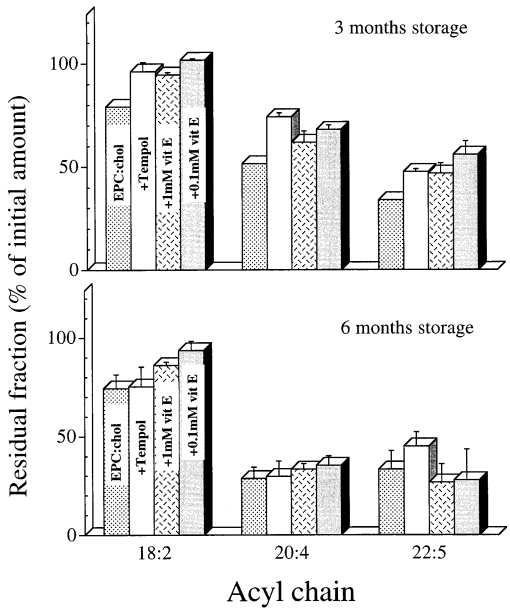

Fig. 2. Effect of antioxidants on damage caused to acyl chain residues of EPC/cholesterol (10:1) liposomes upon long-term storage, exposed to daylight. Liposomal dispersions of EPC/cholesterol (10:1) SUVs in 50 mM Hepes buffer (pH 7.4) were stored at room temperature, for a period of 6 months, with and without either 1 mM Tempol, or 1 and 0.1 mM vitamin E. Acyl chain composition was determined after methyl esterification followed by GC separation. The levels of acyl chains at 3 and 6 months of storage, related to palmitic acid, which served as internal standard, are presented as a fraction of the original levels.

3.4.2. EPC/cholesterol (10:1) liposomes

Degradation of the acyl chain residues of the phospholipids following 3 and 6 months of storage is summarized in Fig. 2. Similar to EPC liposomes, the dispersions that were not exposed to daylight showed similar results (namely, there was no effect of daylight). All acyl chains, including the more sensitive PUFA ($C_{20:4}$ and $C_{22:5}$) showed less degradation than in the case of EPC liposomes (Fig. 1). None of the acyl chains were fully degraded after 6 months of storage. Both Tempol (0.75 mM) and vitamin E (1 and 0.1 mM) provided little protection to PUFA.

3.5. Cholesterol degradation

HPLC analysis revealed that no significant change in cholesterol concentration occurred during 6 months of storage in any of the samples. In addition, no 7-keto-cholesterol, cholesterol's major degradation product, was detected in any of the samples.

3.6. Phospholipid hydrolysis

3.6.1. EPC liposomes

Increase in NEFA concentration in the samples, as a measure of phospholipid hydrolysis, was determined after 16 months of storage, and calculated as the percentage of the total acyl chain content in the liposomal dispersion. The liposomal dispersions at the time of preparation contained NEFA levels of $\sim 0.12\%$ of total acyl chain content. The results are summarized in Table 1. No significant differences among the different daylight-exposed liposomal dispersions were found, all showing NEFA levels of ~ 13–16% of the total phospholipid acyl chains. The

Table 1
Effect of 16 months storage on NEFA content in EPC and EPC/cholesterol (10:1) liposomes with and without antioxidants

Liposomal dispersion	NEFA, % of initial total acyl chain content			
	No antioxidant	+Tempol	+1 mM vitamin E	+0.1 mM vitamin E
EPC				
Light-exposed	16.5 ± 1.5	13.4 ± 1.2	15.9 ± 1.4	
Light-protected	14.6 ± 2	12.6 ± 1.2	12.5 ± 0.5	
EPC/cholesterol (10:1)				
Light-exposed	5.2 ± 0.6	5.4 ± 1.2	3.4 ± 0.4	4.5 ± 0.4
Light-protected	3.9 ± 0.4	3.9 ± 0.5	2.7 ± 0.3	4.1 ± 0.4

Table 2
Residual fraction of nitroxide remaining after 3 and 6 months storage at room temperature of daylight-exposed and -protected samples

Liposomal dispersion	Tempol, residual fraction of initial amount (%)	
	After 3 months	After 6 months
EPC		
Light-exposed		49 ± 9
Light-protected		62 ± 2.5
EPC/cholesterol (10:1)		
Light-exposed	24 ± 1	3 ± 0.3
Light-protected	27 ± 2	7 ± 3

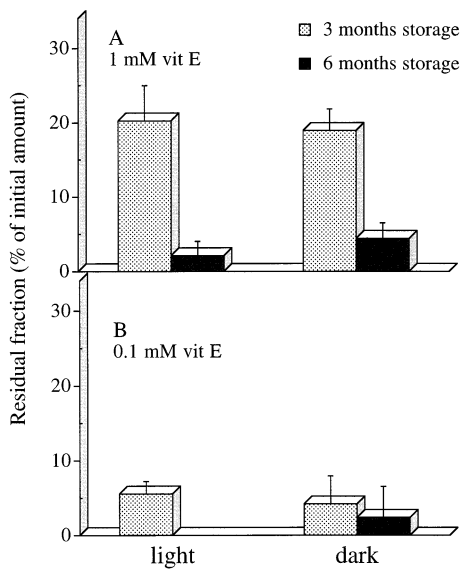

Fig. 3. Degradation of vitamin E upon long-term storage in EPC/cholesterol (10:1) liposomal dispersion. Liposomal dispersions of EPC/cholesterol (10:1) SUVs in 50 mM Hepes buffer (pH 7.4), containing 1 or 0.1 mM vitamin E, were stored at room temperature for a period of 6 months. The samples were either exposed to or protected from daylight. Vitamin E levels were analyzed by HPLC after 3 and 6 months of storage, and are presented as the residual fraction of initial amount in the samples.

daylight-protected samples also show similar, although slightly lower, values (~ 12–14%) of NEFA levels.

3.6.2. EPC/cholesterol (10:1) liposomes

NEFA concentration in the samples was determined after 3, 7, and 16 months of storage, and calculated as the percentage of the total acyl chain content in the liposomal dispersion. All liposomal dispersions showed only a small increase in NEFA (< 1 mol% of the total) after 3 and 7 months of storage. A significant increase in NEFA levels is seen after 16 months of storage, and the results are presented in Table 1. NEFA levels reaching ~ 4–5% of the total phospholipid acyl chains for the daylight-exposed samples, and ~ 3–4% of the total phospholipid acyl chains for the daylight-protected samples.

3.7. Nitroxide (Tempol) degradation

EPR measurements of the samples containing Tempol were made to determine the residual amount of nitroxide in the samples (Table 2). After 6 months of storage, the daylight-exposed and -protected EPC liposomal dispersions showed ~ 50 and $\sim 60\%$ residual fractions, respectively, of Tempol.

EPC/cholesterol liposomal dispersions showed lower residual fractions of Tempol after 6 months of storage. The daylight-exposed and -protected dispersions showed ~ 3 and $\sim 7\%$ residual fractions, respectively, possibly due to the lower initial amount of Tempol in the dispersions (0.75 mM in EPC/cholesterol dispersions and 1 mM in EPC dispersions).

Determination of the total concentration of nitroxide + hydroxylamine (by adding H_2O_2 + NaOH, final concentrations 0.3% and 0.01 N, respectively) in the sample showed that all nitroxide present in the sample was in the oxidized (radical) form.

3.8. Vitamin E degradation

Vitamin E levels in the liposomal dispersions were determined using HPLC and thin-layer chromatography analyses. The results are summarized in Fig. 3. After 3 months of storage, only ~ 4–5% remained in the 0.1 mM vitamin E samples, while $\sim 20\%$ of the initial amount of vitamin E was left in the 1 mM vitamin E samples. After 6

months of storage, the residual fraction of vitamin E was low (~ 0–4%) in all samples. The daylight-protected and -exposed samples showed similar residual amounts of vitamin E after 3 months of storage. After 6 months of storage, the daylight-protected samples contained significantly higher residual amounts of vitamin E than the daylight-exposed samples.

3.9. Effect of cholesterol bilayer levels on Laurdan's GP

Fluorescence intensity of Laurdan at an excitation wavelength of 340 nm and emission wavelength of 440 nm was measured. Laurdan's GP was calculated in EPC/cholesterol liposomes of varying cholesterol concentrations, as detailed in Section 2. As can be seen from the results (Fig. 4), there is an increase both in excitation GP and in emission GP, indicating decreasing bilayer hydration with increasing cholesterol bilayer concentration.

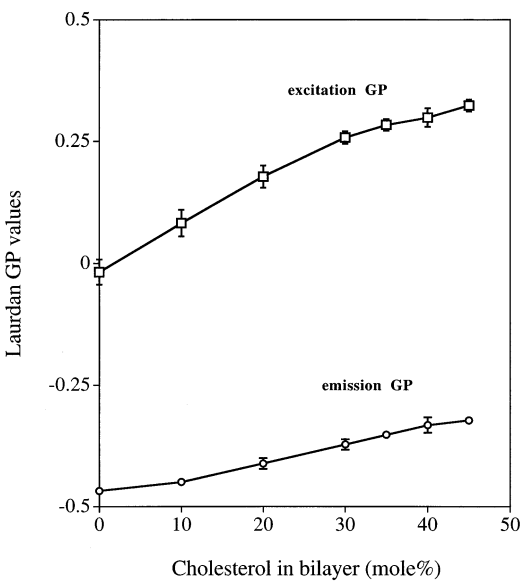

Fig. 4. Effect of bilayer cholesterol on the generalized fluorescence polarization (GP) of Laurdan. Excitation GP_{340} and emission GP_{440} of Laurdan were calculated from the fluorescence measurements of Laurdan-labeled EPC/cholesterol LUV with varying cholesterol concentrations. The change in GP is presented as a function of cholesterol concentration in the bilayer.

4. Discussion

The main aims of the present study were: (1) to investigate the effect of long-term storage on the chemical and physical stability of liposomes prepared of EPC, with and without cholesterol; and (2) to evaluate the protection against liposome degradation provided by cholesterol and antioxidants. The variables studied were: (a) presence of cholesterol; and (b) effect of exposure to, or protection from, daylight. These variables were crossed with the protective effects of two antioxidants, the lipophilic, membrane-residing, α-tocopherol and the water-soluble nitroxide, Tempol.

Vitamin E is a potent, lipid-soluble, chain-breaking antioxidant, both in vivo and in vitro (Burton et al., 1985). Vitamin E refers to one or more of the structurally related phenolic compounds called tocopherols and tocotrienols. We selected α-tocopherol, which has the highest biological activity, as a representative. Vitamin E scavenges the chain-carrying peroxyl radicals rapidly, interrupting the chain reaction propagation. The high activity of vitamin E as an antioxidant stems from its high reactivity toward oxygen-centered radicals, having rate constants of $\sim 10^5$–10^6 $M^{-1}s^{-1}$ (Burton et al., 1985; Niki, 1987). Kinetic studies suggest that one molecule of vitamin E scavenges two molecules of peroxyl radicals (Burton and Ingold, 1981; Niki et al., 1984). Vitamin E suppresses oxidation and produces an inhibition period. It is consumed linearly with time and, when it is depleted, oxidation rate is accelerated to the same rate as that in the absence of α-tocopherol. It has also been reported in studies in vitro, that, under certain conditions, α-tocopherol can act as a pro-oxidant (Cillard and Cillard, 1986; Terao and Matsushita, 1986; Niki, 1987; Yoshida et al., 1994).

Nitroxides are stable, cyclic radicals, which recently have been used to control degradation processes mediated by deleterious reactive species (Nilsson et al., 1989, 1990; Gelvan et al., 1991; Miura et al., 1993; Rachmilewitz et al., 1994). In previous studies, we have clearly demonstrated that Tempol inhibits radiation-induced damage to liposomal lipids, providing protection to all lipids in the bilayer, and to all regions of the lipid

bilayer (Samuni and Barenholz, 1997; Samuni et al., 1997).

4.1. Physical changes in liposomal dispersions

Changes in average particle size and size distribution of liposome dispersions are strongly affected by lipid composition and degree of pH changes. Bilayer structures are destabilized as a result of elevation in NEFA or lysophospholipid concentration. The membranes become more sensitive to aggregation and fusion, and undergo morphological changes (Poole et al., 1970; Lichtenberg and Barenholz, 1988; Grit and Crommelin, 1992, 1993).

EPC/cholesterol liposomes showed no pH or size changes after 6 months of storage, a finding which correlates with the low level of NEFA. A decrease in pH to ~ 7.00 after 16 months of storage also correlates with the increase in NEFA levels then measured.

In EPC liposomes, the changes in size, after 12 months of storage, and in pH, after 16 months, correlate with the increase in NEFA content.

The correlation seen between the physical changes, size and pH, and the increase in NEFA levels indicates that hydrolytic degradation is the major cause of these physical changes. This assumption is supported by the fact, that even though significant PUFA degradation takes place at a much earlier stage of storage, no significant physical changes of the liposomal dispersions are observed.

4.2. Hydrolytic degradation

Lysophospholipids are intermediate products of phospholipid hydrolysis and their concentration can reach a steady-state level while phospholipid hydrolysis continues. For this reason, evaluation of phospholipid hydrolysis cannot be based on determination of lysophospholipids in the liposomal dispersion. The determination of end products, NEFA being one of them, gives a more valid estimation of the hydrolytic degradation. As can be seen from the results obtained, after 6 months of storage, NEFA reached a low level of ≤ 1 mol% of the total initial amount of acyl chains in the dispersion. Only after 16 months of liposome storage did NEFA reach significant levels of 15 and 5 mol% of the total initial amount of acyl chains in EPC and EPC/cholesterol liposomes, respectively. When compared with the results of PUFA degradation, the results of NEFA accumulation indicate that the hydrolytic pathway played a minor role in the chemical degradation that occurred during the long-term storage in this study, as compared with the oxidative pathway. Hydrolytic degradation seems to have a much later onset than the oxidative degradation. The differences in NEFA levels between EPC and EPC/cholesterol liposomes after 16 months of storage point out again the protective effect of cholesterol in the bilayer. These differences in NEFA levels may also account for the difference in pH changes seen between EPC and EPC/cholesterol dispersions.

4.3. Oxidative degradation

In the present study, among all types of liposomes, we selected SUV as analyte, as they show the largest change in size distribution and are the most sensitive to oxidative damage (when compared with multilamellar vesicles; Zuidam et al., 1996; Samuni et al., 1997).

All liposome preparations contained EPC, namely a mixture of egg-derived phosphatidylcholines, including 13.4 mol% PUFA. These PUFA have been found to be the most sensitive region of the phospholipid molecule to radiation-induced oxidative damage (Samuni et al., 1997). As can be seen from Figs. 1 and 2, the sensitivity of PUFA to degradation upon long-term storage is in the order $C_{22:6} > C_{22:5} > C_{20:4} > C_{18:2}$, similar to that previously reported (Samuni et al., 1997). EPC liposome PUFA showed 80–100% degradation after 6 months of storage. The data in Fig. 1 concerning the degradation of $C_{22:5}$ is an experimental result which, in our hands, repeated itself several times. For now, we have no explanation for this result.

The fact that no cholesterol degradation occurred in any of the cholesterol-containing dispersions, even after 6 months of storage, while PUFA showed about 70% degradation, points to

the fact that phospholipid PUFA are the component which 'takes the heat', thereby protecting cholesterol, as previously suggested by Lang and Vigo-Pelfrey (1993). On the other hand, in the presence of cholesterol, PUFA showed less degradation than in the EPC liposomes (lacking cholesterol), which points to the protective effect of cholesterol's presence in the liposomal bilayer. Possibly, due to this protective effect, cholesterol itself is also protected (besides being less susceptible to oxidation to begin with), and therefore shows no degradation.

4.4. *Effect of exposure to, and protection from, daylight*

Daylight-exposed and -protected samples of both EPC and EPC/cholesterol liposomes showed similar results in all the parameters studied: all physical and chemical changes, including both oxidative and hydrolytic degradation. The only differences can be seen in the residual amounts of the antioxidants used in the study, vitamin E and Tempol. Both vitamin E and Tempol showed better survival in the daylight-protected samples, more significantly after 6 months of storage compared with 3 months of storage. It seems that vitamin E and Tempol themselves are depleted as a result of exposure to daylight. However, the similar degradation seen in the samples with no antioxidants seems to indicate that exposure to daylight plays a minor role in the chemical stability of liposome lipids during storage.

4.5. *Comparing protective effects of vitamin E and Tempol*

PUFA were proved in this study, as well as in others, to be the most sensitive part of the liposome to degradation, and the protective effect of antioxidants against PUFA oxidation was most pronounced.

In the case of EPC liposomes, which were clearly more sensitive to degradation than cholesterol-containing liposomes, Tempol, in a concentration of 1 mM, was a better antioxidant, and provided significantly better protection to PUFA, than 1 mM vitamin E. In cholesterol-containing liposomes, 0.75 mM Tempol had no advantage over either 1 or 0.1 mM vitamin E, possibly since the presence of cholesterol in the bilayer played a dominant role in protecting.

Vitamin E at 0.1 mM was not a worse, maybe even a slightly better, protectant than at a concentration of 1 mM, a fact that can be explained by the pro-oxidative properties of vitamin E at higher concentrations, as reported in several studies (Cillard and Cillard, 1986; Terao and Matsushita, 1986; Niki, 1987; Yoshida et al., 1994).

Both antioxidants seem to have no effect on hydrolytic degradation occurring at a much later stage of storage. This could be due either to their mechanism of action, or to the fact that most antioxidants are depleted at the time of hydrolytic degradation onset.

The fact that in EPC liposomes the residual fraction of Tempol is higher than that of vitamin E can be explained by the unique recycling of piperidinyl nitroxides (Krishna et al., 1992). The recycling mechanism of action is a great advantage of Tempol over stochiometrically acting vitamin E, which is almost or completely consumed after 6 months of storage (depending on initial concentration and exposure to daylight). This recycling mechanism can be the reason for Tempol's better protection of EPC liposomes.

Tempol, like other nitroxides, has lower bimolecular rate constants with oxygen-centered radicals ($\sim 10^3$ to 10^4 $M^{-1}\cdot s^{-1}$) than vitamin E, but it readily reacts with carbon-centered radicals ($\sim 10^8$ to 10^9 $M^{-1}\cdot s^{-1}$) to give stable, non-radical products (Kocherginsky and Swartz, 1995).

4.6. *Effect of bilayer cholesterol on liposomal degradation during storage*

The results presented in this study clearly show that EPC liposomes were more sensitive to both oxidative and hydrolytic degradation during long-term storage than EPC/cholesterol liposomes. This indicates that the presence of cholesterol in the bilayer has a protective effect. We selected the EPC/cholesterol ratio of 10:1 to be in the range where protection is only partial and not complete, which will allow us detection of the effect of the other additives in our study.

Cholesterol has been shown previously to have antioxidative properties in biological membranes and liposomes (Bereza et al., 1985; Clemens and Waller, 1987; Parasassi et al., 1995b). The presence of cholesterol in the lipid bilayer decreases the mobility of the molecules and reduces bilayer permeability. It modifies the physical state from the liquid-disordered (LD) (fluid) phase (or from solid-ordered (SO) phase) to an intermediate phase, the liquid-ordered (LO) phase, intermediate in degree of chain order between LD and SO phases (Mouritsen, 1991). This increase in order and rigidity of the acyl chain region of bilayers enriched with unsaturated acyl chains was proposed as a mechanism for increasing the resistance to in vivo liposome degradation (Lasic, 1993). The protective effect of cholesterol was related to its influence on the structure and dynamics of the bilayer: (1) increasing the order of bilayer enriched with unsaturated acyl chains, promoting tighter packing of the bilayer; and (2) causing steric hindrance to the diffusion of the radical chain propagation reaction because of cholesterol's presence between two adjacent unsaturated acyl chains (Parasassi et al., 1995b).

Our study suggests an additional dimension to the protection by cholesterol. As demonstrated before, and confirmed in this study, cholesterol 'dries' the lipid; its presence in lipid bilayers has been shown to decrease the hydration of the bilayer. This effect has been related to the general effect of cholesterol of increasing the bilayer packing in the headgroup and acyl chain regions, decreasing the rate of diffusion of water into the bilayer, its depth of penetration, and its concentration in the bilayer (Simon et al., 1982; Parasassi et al., 1994a,b, 1995a). As can be seen from our results, EPC/cholesterol liposomes with varying cholesterol concentrations demonstrate an increase in Laurdan's GP values with the increase in cholesterol concentration (Fig. 4). The increase in GP values reflects a decrease in bilayer hydration.

Water has been shown to play a major role in radiation-induced oxidative damage, with the aqueous phase being the source of reactive oxygen species (ROS) involved (Samuni and Barenholz, 1997; Samuni et al., 1997; Tirosh et al., 1997). Therefore, it has been suggested that reducing the content of water associated with the lipid bilayer should influence resistance of liposomes to oxidative damage and increase their stability (Priev et al., 1998).

Water at physiological conditions (in the absence of γ-irradiation) does not form radicals, nor does it react with radicals formed during lipid peroxidation (LPO) reactions. Still, water can indirectly have a large effect in accelerating LPO. It can cause hydrolysis of metals, metal complexes, or other radical species formed during the oxidation process in proximity to the lipid bilayer, and thus form other, more reactive species, enhancing increased damage. Cholesterol's protective effect might then be a result of decreasing bilayer hydration and decreasing the content and the molecular dynamics of water molecules present at the hydrophobic–hydrophilic interface of the bilayer. The outcome is decreasing the source and the mobility of ROS and intermediates of lipid peroxidation within the headgroup/acyl chain interface and in the acyl chain region of the lipid bilayer. Furthermore, decrease in water content and, consequently, protons and hydroxide ions, results in a decrease of direct hydrolytic degradation caused to the membranal lipids.

5. Conclusions

1. EPC liposomes are more sensitive to degradation during storage than EPC/cholesterol liposomes. Cholesterol in the lipid bilayer has a protective effect, mainly due to decreasing lipid-bilayer hydration.
2. PUFA are the most sensitive components of the liposome bilayer to oxidative degradation during long-term storage.
3. Oxidative degradation has a much earlier onset than hydrolytic degradation.
4. Tempol provides significantly better protection than vitamin E to EPC liposomal PUFA against degradation during long-term storage.

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