Novel synthetic phospholipid protects lipid bilayers against oxidation damage: role of hydration layer and bound water†

Oren Tirosh,[a] Ron Kohen,[a] Jehoshua Katzhendler,[b] Raphael Gorodetsky[c] and Yechezkel Barenholz*,[d]

[a] *Department of Pharmacy, School of Pharmaceutics, The Hebrew University of Jerusalem, Israel*
[b] *Department of Pharmaceutical Chemistry, School of Pharmacy, The Hebrew University of Jerusalem, Israel*
[c] *Sharett Institute of Oncology, Hadassah University Hospital, Jerusalem, Israel*
[d] *Department of Biochemistry, The Hebrew University–Hadassah Medical School, PO Box 12272, Jerusalem 91120, Israel*

The role of the membrane hydration layer in preventing membrane oxidative damage has been evaluated by comparing bilayers with and without an extended hydration layer. The extended hydration layer was obtained through the use of a novel phospholipid in which polyethylene glycol of 2000 Da molecular mass (PEG^{2000}) was covalently attached to the phosphate headgroup of a phospholipid backbone to form dihexadecylphosphatidyl PEG^{2000}—α-{[2,3-bis(hexadecyloxy)propoxy](hydroxyphosphinoyl}-ω-methoxypoly(oxyethane-1,2-diyl) monosodium salt. The amount of water bound to free PEG and to the DHP-PEG^{2000} was determined by differential scanning calorimetry. Small unilamellar liposomes composed of egg phosphatidylcholine and DHP-PEG^{2000} were prepared. 44% of the phospholipid contained one polyunsaturated acyl chain. Oxidative damage to liposomes after exposure to three different oxidation procedures was measured by the disappearance of polyunsaturated acyl chains, as determined by GC. Oxidation procedures used were: (*i*) exposure to ionizing γ-irradiation (9200 Gy), for which the grafted PEG^{2000} provided significant protection against oxidation, with minimal damage to the PEG^{2000} as determined by ^{1}H NMR and TLC. (*ii*) Storage for 6 months at 4 °C or for 4 months at 4 °C followed by 4 d at 37 °C, for which the presence of DHP-PEG^{2000} retarded acyl chain peroxidation. (*iii*) Oxidation of the liposomes by 2,2′-azo(2-amidinopropane) dihydrochloride (a positively charged water-soluble peroxyl radical initiator), for which there was no protection by DHP-PEG^{2000} (probably due to electrostatic binding of the AAPH to the negatively charged membranes, thereby overriding the hydration layer protection barrier).

Introduction

Water bound tightly to model bilayer membranes, or to cell membranes, differs from normal bulk water in that it is not active osmotically and possesses low compressibility similar to ice. Therefore it affects the movement, distribution and organization of many species near the membrane surfaces.[1,2] Our aim in this study was to elucidate the contribution of bound water to improve liposome resistance to oxidative damage.

Oxidation processes in membranes are initiated by reactive oxygen species forming in the aqueous phase and in the lipids, leading to oxidative degradation of polyunsaturated phospholipid acyl chains in the lipid bilayer.

Peroxidation and autooxidation of organic compounds are free radical chain processes in which three major stages are recognized: initiation, propagation and termination.[3] A large fraction of the acyl chains of the lipid of membranes surrounding cells and subcellular organelles are polyunsaturated fatty acids (PUFA) and therefore sensitive to oxidative processes, as is membrane cholesterol. What therefore, protects these molecules? Barclay and Ingold[3] have demonstrated that the membrane lipid assembly itself has preventative antioxidant properties: oxidizability of membrane lipids is inhibited by 97%, compared to lipids which are dispersed in homogeneous solutions of organic solvents.[3] The anisotropic organization, tight packing and interaction between its lipidic membrane components, together with water surrounding the lipid bilayer, are given as the main protecting factors.[3]

Biological membranes are more complex systems than simple lipid bilayers. They are composed of a large variety of lipids, proteins, antioxidants and glycocalyx (composed of glycolipids and glycoproteins), which contribute to the hydration layer surrounding the cell.[4] We propose that this layer may be a barrier against oxidative damage. To test the hypothesis that tightly bound water protects against peroxidation, we compared the oxidizability of liposomes in the absence and presence of an extended hydration layer.

Dihexadecylphosphatidyl polyethyleneglycol^{2000}, a new synthetic highly stable phospholipid molecule having polyethylene glycol of molecular mass 2000 Da (PEG^{2000}) as part of its headgroup was used to increase the hydration layer. The selection of this lipid was based on (*i*) recent studies which demonstrated that PEG^{2000}, when attached to the primary amine headgroup of phosphatidylethanolamine, acts in liposomes as a steric stabilizer, thereby prolonging circulation time of liposomes *in vivo*;[5] and (*ii*) previous data on the very high capacity of PEG to bind water.[6] The present study showed that PEG^{2000} when grafted in the lipid bilayer increases the level of hydration and results in protection of the bilayer PUFA against oxidative damage, suggesting that the hydration layer serves as a barrier against oxidative damage initiated in aqueous medium.

† *Abbreviations:* PEG^{2000}, polyethylene glycol of molecular mass 2000 Da; DHP-PEG^{2000}, dihexadecylphosphatidyl-polyethylene glycol 2000; PE, phosphatidylethanolamine; PC, phosphatidylcholine; ROS, reactive oxygen species; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; DSC, differential scanning calorimetry; HC, 4-heptadecyl-7-hydroxycoumarin; PUFA, polyunsaturated fatty acid; AAPH, 2,2′-azo(2-amidinopropane) dihydrochloride.

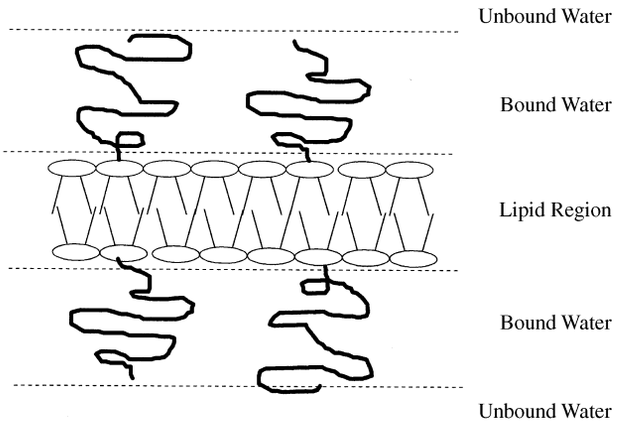
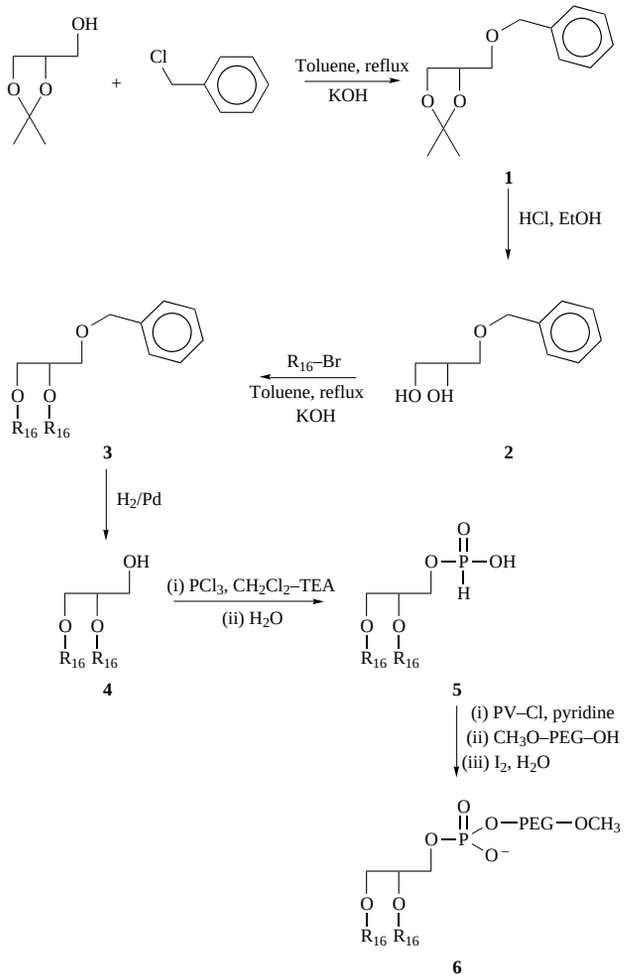


Fig. 1 Diagram of membrane having an extended hydration layer due to PEG^{2000} grafted on the lipid bilayer surface



Scheme 1 Synthesis of DHP-PEG^{2000}. The synthesis was carried out using the H-phosphate strategy as described in the Experimental section.[14,15]

Results

Synthesis of PEG lipid

The racemic DHP-PEG^{2000} was synthesized (Scheme 1) and characterized by ^{31}P NMR, ^{1}H NMR, IR, TLC and element analysis, as described in the Experimental section.

Effect of PEG^{2000} and DHP-PEG^{2000} on differential scanning calorimetry (DSC) measurements in HEPES–saline solution

Increasing concentrations of PEG^{2000} in HEPES–saline (20 mM HEPES buffer, pH 7.2, in 150 mM NaCl) solution induced dramatic changes in the 'heat flow DSC ΔH_{fu}' plot of water. Fig. 2 is a typical scan of the HEPES–saline solution with a

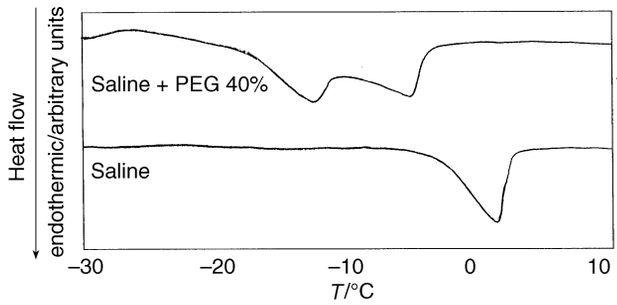


Fig. 2 Effect of 40% PEG^{2000} on the ΔH_{fu} of water in HEPES–saline solution (upper thermogram). ΔH_{fu} of water in HEPES–saline (lower thermogram). The heat flow profile of HEPES–saline was changed in the presence of PEG^{2000}. Two peaks were detected, at −5 °C and −12 °C. In HEPES–saline only one peak was detected, at 2 °C.

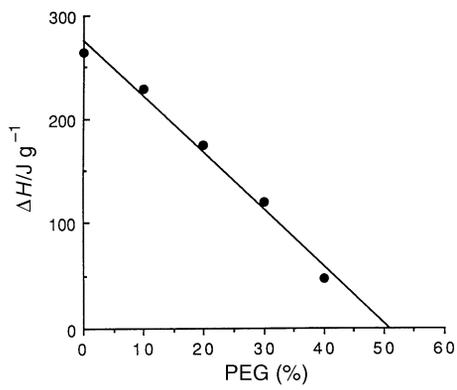


Fig. 3 Effect of PEG^{2000} concentration on ΔH_{fu} of water in HEPES–saline solutions of PEG^{2000}

single peak of water melting at *ca.* 0 °C. The scan was performed from −30 to 10 °C for pure HEPES–saline and for HEPES–saline solution containing increasing concentrations of PEG^{2000}. ΔH_{fu} was calculated from the area of the peak to be 275 J g^{-1} for the water with HEPES–saline. 40% (w/w) of PEG^{2000} induced almost complete disappearance of the free water peak (Fig. 2). This is an indication that water became tightly bound to the PEG molecules. ΔH_{fu} of the water was calculated in the presence of 40% PEG and 150 mM NaCl to be 49 J g^{-1}. From a calibration curve of the ΔH_{fu} of water *vs.* PEG concentrations (Fig. 3) it was calculated that each molecule of PEG binds 136 molecules of water. Similar studies were carried out for DHP-PEG^{2000} micelles dispersed in HEPES–saline at a concentration of 10% (w/w). ΔH_{fu} of the water at such a DHP-PEG^{2000} concentration was found to be 210 J g^{-1}. The number of water molecules bound to each DHP-PEG^{2000} molecule in the micellar solution was calculated to be 197. In order to compare DHP-PEG^{2000} to a conventional phospholipid containing a phosphocholine headgroup, we added 3.2 mg of HEPES–saline to 4 mg of dimeristoylphosphatidylcholine. Complete hydration of the phospholipid was achieved by freeze–thawing the mixture four times. From the heat flow of the mixture thermogram, ΔH_{fu} of the water was reduced to 165 J g^{-1} and the number of water molecules bound to a dimeristoylphosphatidylcholine lipid molecule was calculated to be ten.

Irradiation experiment

Liposomes containing and lacking DHP-PEG^{2000} were exposed to ionizing γ-irradiation. Liposomes were analysed for their acyl chain composition before and after exposure to γ-irradiation, as described in the Experimental section. Three species of PUFA were followed (18:2, 20:4, 22:6). The internal standard used to calibrate loss of PUFA in the liposomes was the saturated 16:0 fatty acid, palmatic acid, which was unaffected by the irradiation. Liposome acyl chain composition before irradiation showed no differences in fatty acid composition between the

Table 1 PUFA/16:0 ratio in liposomes having and lacking DHP-PEG^{2000}, before and after exposure to 9200 Gy γ-irradiation[a]

	Egg-PC liposomes			Egg-PC/DHP-PEG^{2000} liposomes		
PUFA	18:2	20:4	22:6	18:2	20:4	22:6
Before irradiation	0.527 ± 0.008	0.087 ± 0.003	0.043 ± 0.004	0.530 ± 0.008	0.090 ± 0.006	0.045 ± 0.004
After irradiation	0.450 ± 0.01	0.048 ± 0.005	0.019 ± 0.002	0.487 ± 0.011	0.073 ± 0.006	0.032 ± 0.005

[a] $n = 6$, $p < 0.05$.

Table 2 Effect of AAPH on surface potential (ψ_0) as assessed from the apparent pK_a of HC in liposomes[a]

	Egg-PC liposomes	Egg-PC/ DHP-PEG^{2000} liposomes	Egg-PC liposomes +10 mM AAPH	Egg-PC/DHP-PEG^{2000} liposomes +10 mM AAPH
pK_a	9.3	10.25	9.4	9.5
ψ/(mV)	0	−56.1	−5.9	−11.8

[a] Apparent pK_a of 4-heptadecyl-7-hydroxycoumarin (HC) incorporated in liposomes composed of egg-PC and liposomes composed of egg-PC/DHP-PEG^{2000} at a ratio of 1:200 HC:lipid (liposome lipid concentration was 0.31 mM). Surface potential was calculated from $-pK_{aSurface} = -9.3 + (\psi_0 e)/(kT \ln 10)$. For more details see Experimental section and ref. 19.

two types of liposomes, as shown in Table 1. γ-Irradiation at a dose of 9200 Gy caused a significant loss of PUFA in all of the liposomes. As expected, the loss of PUFA increased with increasing degree of phospholipid acyl chain unsaturation for liposomes lacking and containing DHP-PEG^{2000}. However, the level of oxidative damage was much higher for the vesicles lacking PEG, especially for 20:4 and 22:6 acyl chains (Table 1).

Evaluation of oxidative damage to PEG^{2000} moiety of the DHP-PEG^{2000}

The lipids of the irradiated liposomes were extracted into the chloroform-rich phase. It was expected that the hydrophilic fragments and degradation products of the polyethylene glycol resulting from the exposure to γ-irradiation, if formed, would partition into the aqueous phase together with other water-soluble components, while the intact PEG and its lipophilic derivatives should be extracted into the organic phase together with the egg-PC. More than 95% of the phospholipid (phosphorus determination) and the PEG^{2000} (^{1}H NMR) were recovered in the chloroform-rich phase, indicating that the PEG was still covalently attached to the lipid. ^{1}H NMR showed that damage to the DHP-PEG^{2000} was minimal. Comparison between integration of the peaks at 3.35 ppm (methyl headgroup of choline) and the peaks at 3.7 ppm (polyethylene glycol methylene groups) before and after 9200 Gy irradiation in six separate experiments, showed that the change in the integration ratio was minimal. The mean loss of PEG in these experiments was 6.4 ± 3.8%. The loss of PEG, if indeed it occurs, was very small. Following 9200 Gy irradiation, ^{1}H NMR spectra did not reveal any choline loss while changes in the acyl chains occurred.

Oxidation of the PEG moiety may result in accumulation of peroxides. This was studied in liposomes composed of two saturated phospholipids (a 1:1 mixture of dimeristoylphosphatidylcholine and dimeristoylphosphatidylglycerol) containing 6 mol% DPH-PEG^{2000}. In order to maximize the damage to the PEG moiety the liposomes were irradiated for 54 h with a total dose of 27 500 Gy. No accumulation of peroxides was found in the PEG-lipid molecules following irradiation. Finally, TLC testing of the liposome preparations following irradiation revealed a single spot having R_f identical to the original DHP-PEG^{2000}.

AAPH oxidation of liposomes

Liposomes (8 mM phospholipids) with and without DPH-PEG^{2000} were incubated with and without (control) AAPH (20 mM) at 37 °C for 24 h, then peroxides accumulation was determined. In the presence of AAPH both egg-PC liposomes and egg-PC/DHP-PEG^{2000} liposomes significantly accumulated peroxides. The liposomes containing DHP-PEG^{2000} have a higher level of peroxides than liposomes composed only of egg-PC (Fig. 4). Parallel measurements of acyl chain composition revealed a complete loss of PUFA after 72 h incubation in both

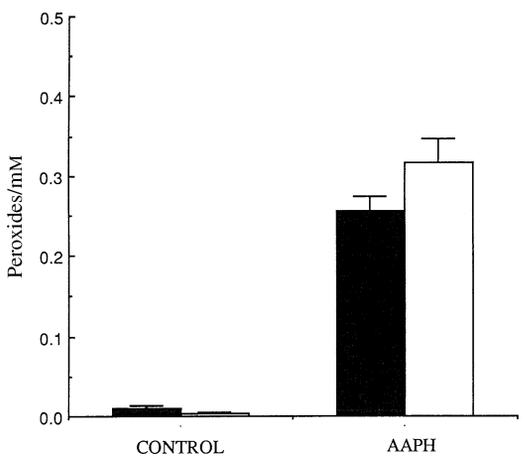


Fig. 4 Accumulation of lipid peroxides in liposomes composed of egg-PC (black columns) and in liposomes composed of egg-PC/DHP-PEG^{2000} (white columns) following 24 h of incubation in AAPH (20 mM) at 37 °C *vs.* control of 24 h at 37 °C ($n = 4$, $p < 0.05$)

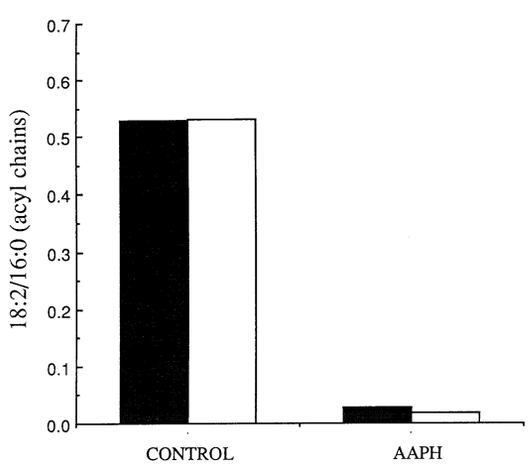


Fig. 5 Acyl chain (18:2) loss in liposomes composed of egg-PC (black columns) and in liposomes composed of egg-PC/DHP-PEG^{2000} (white columns) following exposure to AAPH (20 mM) at 37 °C for 72 h *vs.* control of 72 h at 37 °C. Average of $n = 2$; the difference between the two experiments was less than 6% of the average.

Table 3 Loss of acyl chains 20:4 and 22:6 due to oxidation following 4 d incubation at 37 °C under air in two liposome preparations[a]

	Egg-PC liposomes		Egg-PC/DHP-PEG^{2000} liposomes	
PUFA	20:4	20:6	20:4	20:6
Loss (%)	19	27	5	6

[a] $n = 2$, $p < 0.05$.

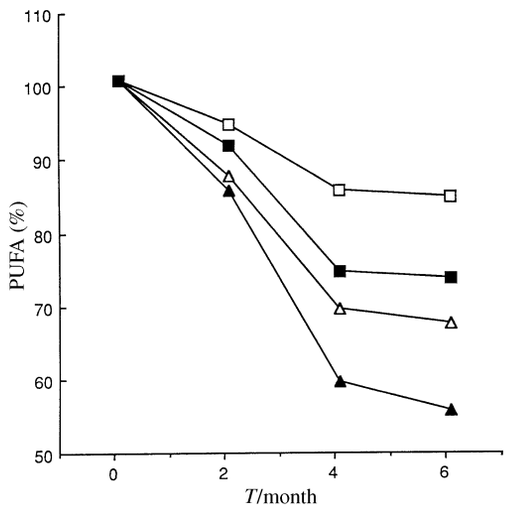


Fig. 6 Kinetics of loss of acyl chains 20:4 and 22:6 with storage time up to 6 months at 4 °C under air in two liposome preparations. Empty symbols represent liposomes composed of egg-PC/DHP-PEG^{2000}; filled symbols, liposomes composed of only egg-PC; squares, loss of 20:4 acyl chain; triangles, loss of 22:6 acyl chain. Results represent the mean of two separate experiments ($p > 05$).

preparations (Fig. 5). The explanation for the accelerated oxidative damage is given below.

AAPH adsorption on liposome surface

The electrical surface potential (Ψ_0) of the egg-PC and egg-PC/DPH-PEG^{2000} (97:3, mol:mol) liposomes was determined in the absence and presence of AAPH by including the pH-sensitive fluorescent probe 4-heptadecyl-7-hydroxycoumarin (HC) at the liposome membrane (0.31 mM lipids). Table 2 demonstrates that the DPH-PEG^{2000} introduced negative electrical surface potential to the liposome surface and that this was almost completely neutralized in the presence of 10 mM AAPH, as was assessed from the shift in the ionization curve of HC in the lipid bilayer to lower bulk pH. This indicates electrostatic interaction between the DHP-PEG^{2000} liposomes and AAPH (Table 2).

Liposome oxidation after long exposure to air

The degree of oxidation of the liposome lipids after 6 months storage at 4 °C was measured by changes in aycl chain composition. PUFA of the egg-PC/DHP-PEG^{2000} preparation were found to be more resistant to oxidation than PUFA of the liposomes composed of egg-PC only. Liposomes containing DHP-PEG^{2000} lost 16 and 33% of their 20:4 and 22:6 acyl chains, respectively, compared with a loss of 27 and 45%, respectively, in liposomes lacking DHP-PEG^{2000} (Fig. 6). Liposomes containing and lacking DHP-PEG^{2000} were stored for 4 months at 4 °C and then for 4 d at 37 °C, and then the relative loss of PUFA was measured. Under accelerated oxidation at 37 °C the loss was 19 and 27% for the 20:4 and 22:6 acyl chains, respectively, for vesicles lacking DHP-PEG^{2000}, while the loss in the DHP-PEG^{2000} liposomes was more limited (5 and 6% for the 20:4 and 22:6 fatty acids, respectively) (Table 3).

Discussion

The oxidative damage to the membrane lipids and proteins by free radicals and other reactive oxygen species (ROS) is a well established phenomenon. It is initiated when a strong oxidizing agent interacts directly with the lipid and abstracts hydrogen atoms from the bisallyl position in the acyl side chain. From the simple electrochemical perspective, many radicals may have this ability to initiate lipid peroxidation,[7,8] yet we know that, in general, the initiation process has very low efficiency (defined as the fraction of radicals inducing chain oxidation).[3] In a nonhomogeneous, nonisotropic assembly of a biological membrane or a lipid bilayer the efficiency of initiation is reduced due to the nonisotropic tight packing of the lipid molecules, which acts as a barrier against penetration of radicals into the bilayer, thereby reducing the availability of the polyunsaturated acyl chains. The packing energy in the polar headgroup region is in the range 20–50 mJ m^{-2}, depending on the attractive interaction arising mainly from the hydrophobic chains.[9] Most, if not all, of the free radicals which initiate the peroxidation originate in the water. Initiators of free radicals such as radiation or enzymes (*e.g.* xanthine oxidase) all produce their radicals in the water surrounding (or within) living cells. Therefore, the elucidation of the process of initiation has to take into consideration the diffusion of the radical until it reaches and penetrates the membrane.

Propagation of a free radical chain reaction is also strongly inhibited by the bilayer structure. The peroxyl radical possesses a strong dipole moment and the driving force of its movement is from the inside of the membrane towards the water (a downhill trajectory).[8]

This work was aimed at determining the role and contribution of the contiguous water around the membrane in controlling oxidation. The hydroxyl radical has a very short half-life ($t_{\frac{1}{2}}$ of *ca.* 10^{-12} s)[10] and it can therefore diffuse only for short distances. Therefore even small changes in the hydration layer may affect membrane lipid oxidation.

We used three different ways to initiate oxidative damage: (*i*) γ-irradiation, which ionizes and excites water molecules;[10,11] (*ii*) the positively charged hydrophilic azo compound AAPH, which generates a constant flux of peroxyl radicals at a temperature-dependant rate;[12] (*iii*) time-dependant autooxidation in air.

In order to enable selective measurement of the stability of the phosphatidylcholine (which is an ester phospholipid) against lipid peroxidation, the PEGylated molecule was prepared as a saturated lipid in which the hydrophobic chains were connected to the glycerol *via* ether bonds to form the DHP-PEG^{2000} (Scheme 1). The oxidative damage to the PC acyl chains was determined using GC.[10,17]

γ-Irradiation of water leads to the formation of various free radicals and ROS including hydroxyl radicals, electrons, hydrogen atoms and superoxide radicals.[10,11] Recently, we obtained direct evidence that scavenging these ROS in the aqueous phase by stable nitroxide radicals completely inhibits the oxidative damage to liposomal lipid acyl chains, indicating that ROS formed by γ-irradiation in the aqueous phase are the source of oxidative damage.[20] These radicals must diffuse into the membrane in order to initiate lipid oxidation. Here, we demonstrate that PEG^{2000} covalently attached to a phospholipid strongly reduces oxidative damage from γ-irradiation with minimal or no damage to the PEG^{2000} moiety. This lack of damage to the PEG^{2000} indicates that its protective effect is not due to its activity as a radical scavenger. We propose that its protective effect is related to its capacity to bind tightly large amounts of water to the membrane surface. These bound water molecules are not available as a source of ROS, and also ROS formed in the unbound water have further to go to reach the membrane, while the bound water imposes slower motion towards the membrane surface. A relatively low mole fraction (3 mol%) of DHP-PEG^{2000} in a lipid bilayer is sufficient to form a protective barrier of bound water (increasing the hydration layer by 40%). This protection can be viewed as a radical 'freezing' mech-

anism. Indeed, freezing has been shown to reduce oxidative damage to liposomes following irradiation.[13]

To test further this hypothesis we induced ROS formation near the phosphate group of membrane phospholipids, overriding the steric barrier of the PEG^{2000}. This was done by AAPH, a positively charged small molecule which we proved was absorbed to the negatively charged membrane phosphate (Table 2). Thermal decomposition of AAPH continuously produced peroxyl radicals near the phospholipid ester bond region without the need to diffuse through the hydrated headgroups. The vacant site in the membrane formed after AAPH decomposition was then occupied by a new AAPH molecule (in equilibrium with bulk AAPH). This mechanism permits the oxidation of the membrane starting from the interface region of the bilayer, thus overcoming the protection by the grafted PEG. Egg-PC/DHP-PEG^{2000} liposomes were even more susceptible to oxidation by AAPH than egg-PC liposomes, as a consequence of the site-specific adsorption of AAPH on the liposome surface due to the negatively charged DHP-PEG^{2000}, as was demonstrated by the change in electrical surface potential (Table 2).

The oxidation of the liposomes upon long storage in air without any protective or preventive measures is the result mainly of propagation of the oxidation process in the liposome lipids. The PUFA of PEG^{2000}-containing liposomes were more resistant to oxidative damage than those lacking PEG during 6 months storage at 4 °C, although significant loss of PUFA also occurred. Almost complete protection of the liposomes containing grafted PEG^{2000} was obtained at 37 °C, a temperature at which the initiation and the propagation steps were accelerated (Table 3).

It is concluded that an extended hydration layer surrounding the lipid–water interface may be an effective physical mechanism to prevent oxidation of membranes. The hydration layer affects the oxidation probably by inhibition of ROS formation and movement and free radical formation in close proximity to the bilayer, thus affecting both propagation and initiation steps in oxidation processes.

This novel protection mechanism may be of great importance in improving liposome chemical stability and protecting biological external tissues exposed to the atmosphere, such as skin and eyes, against oxidative damage from endogenous and exogenous sources of ROS, including inflammation and irradiation. The increase in hydration layer of tissues may be achieved by topical applications of advanced pharmaceutical formulations based on liposomes or emulsions containing lipids such as DHP–PEG^{2000}.

The glycoside-rich glycocalyx in biological membranes may play a role similar to that of DHP-PEG^{2000} in protecting biological membranes against oxidative damage.

Experimental

General

NMR spectra were recorded in $CDCl_3$ solution on a Varian VXR 300S spectrometer. Chemical shifts are reported in ppm downfield from $SiMe_4$ as internal standard in ^{1}H spectra (300 MHz) and from 1% H_3PO_4 in D_2O as external standard in ^{31}P spectra (121 MHz). Positive chemical shifts are at low field with respect to the standard. All reagents for the synthesis were of analytical grade or better (Aldrich, Milwaukee, WI, USA).

3-*O*-Benzyl-1,2-*O*-isopropylideneglycerol (1)

To a solution of 1,2-*O*-isopropylideneglycerol (solketal) (10 g, 0.075 mol) and benzyl chloride (13 g, 0.092 mol) in toluene (500 ml) was added powdered potassium hydroxide (100 g) and the mixture was refluxed with stirring for 16 h, with removal of the water formed using a Dean–Stark device. After completion of the reaction, water (500 ml) was added to dissolve the inorganic salts. The phases were separated, the toluene phase was washed three times with water, dried over magnesium sulfate and evaporated under reduced pressure. Distillation of the residue yielded the ether **1** (14.6 g, 0.066 mol, 88%), bp 89 °C at 0.15 mmHg. δ_H 1.2 (s, 3 H), 1.28 (s, 3 H), 3.3–3.5 (m, 2 H), 3.6–3.7 (m, 1 H), 3.9–4.1 (m, 1 H), 4.1–4.25 (s, 1 H), 4.4–4.6 (s, 2 H), 7.1–7.3 (m, 5 H).

3-*O*-Benzylglycerol (2)

A solution of the ether **1** (10 g) in a mixture of ethanol (100 ml) and concentrated hydrochloric acid (10 ml) was refluxed while monitoring the progress of the reaction by TLC. After 1 h reflux the cleavage of the isopropylidine group was complete, ethanol was evaporated, 200 ml of water was added and the product was isolated by freeze-drying the solution. The liquid was not purified further and the reaction continued from the same pot.

1,2-Di-*O*-hexadecyl-3-*O*-benzylglycerol (3)

A mixture of 3-*O*-benzylglycerol (3.46 g, 0.019 mol), 1-bromohexadecane (24.4 g, 0.08 mol) and potassium hydroxide (11.2 g) in toluene (200 ml) was refluxed with stirring for 16 h, with removal of the water formed using a Dean–Stark device. The cooled mixture was washed three times with water, dried over magnesium sulfate, and the toluene was evaporated under reduced pressure. The residue was distilled under vacuum to remove unreacted hexadecyl bromide, bp 105 °C at 0.15 mmHg. The residual oil (9 g, 0.014 mol, 75%) was not purified further. δ_H 0.8–1 (t, 6 H), 1.2–1.4 (s, 52 H), 1.5–1.7 (m, 4 H), 3.3–3.7 (m, 9 H), 4.6 (s, 2 H), 7.3 (m, 5 H).

1,2-Di-*O*-hexadecylglycerol (4)

1,2-Di-*O*-hexadecyl-3-*O*-benzylglycerol (**3,** 5 g) dissolved in warm butanol (200 ml) was hydrogenated by shaking in the presence of 1 g of 10% Pd/C under 45 psi H_2‡ for 10 h. The mixture was then diluted with 300 ml of chloroform and filtered to remove the catalyst. The combined filtrate was evaporated under reduced pressure to dryness. The solid residue was recrystallized from 40 ml acetone to yield 4 g (0.0074 mol, 93%) of product. δ_H 0.8–1 (t, 6 H), 1.2–1.4 (s, 52 H), 1.5–1.7 (m, 4 H), 3.4–3.8 (m, 9 H).

Pyridinum 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphonate (pyridinum salt of 5)

To a stirred solution of phosphorus trichloride (1.25 g, 0.009 25 mol) in dichloromethane (100 ml), a solution of 1,2-di-*O*-hexadecylglycerol (**4,** 1 g, 0.0018 mol) and triethylamine (TEA; 1 g, 0.0099 mol) in 20 ml of dichloromethane was added dropwise for 20 min. Stirring was continued for 30 min and the reaction mixture was quenched by addition of 100 ml of water–pyridine (1:4, v/v). After 15 min, 300 ml of chloroform was added and the organic layer was washed twice with 100 ml of water, dried with sodium sulfate and evaporated under reduced pressure. The solid residue was crystallized in acetone. Yield 1 g (0.001 46 mol, 81%). NMR δ_P 1.2–7.0 (dt, *J* 665). δ_H 0.8–1 (t, 6 H), 1.2–1.4 (s, 52 H), 1.5–1.7 (m, 4 H), 3.3–3.7 (m, 7 H), 3.9–4.1 (m, 2 H).

Sodium 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phospho-PEG^{2000} monomethyl ether (6) (DHP-PEG^{2000}

Compound **5** (1 g, 0.001 46 mol) was dissolved in 50 ml of dichloromethane. Lyophilized polyethylene glycol monomethyl ether (CH_3–O–PEG^{2000}–OH; 3.5 g) pivaloyl chloride (0.35 g, 0.0029 mol) and pyridine (1 ml) were added to the reaction mixture. After 10 min of stirring, the reaction mixture was evaporated to dryness under reduced pressure. A solution of 0.8 g of I_2 in 15 ml of water–pyridine (1:1, v/v) was added in order to oxidize the *H*-phosphonate. Oxidation took place for 10 min and was stopped by addition of 100 ml of 5% aqueous sodium thiosulfate solution. The lipid was extracted from the aqueous

‡ 1 psi = 6895 Pa.

medium with 200 ml of chloroform. The organic layer was separated, dried over magnesium sulfate, filtered and evaporated under reduced pressure. The solid residue was crystallized from acetone, mp 49–50 °C. For comparison, mp of methoxy-PEG^{2000} (the precursor used for DHP-PEGylation) is in the range of 54–56 °C, while the mp of DHP is above 150 °C. The melting points of the above compounds indicate that the PEG moiety dominates the melting behaviour of DHP-PEG^{2000}. Yield 3.5 g (0.001 29 mol) 88%. δ_H 0.7–0.8 (t, 6 H), 1.2–1.4 (s, 52 H), 1.45–1.6 (m, 4 H), 2.3–2.5 (m, 9 H), 3.5–3.8 (s, 181 H), δ_P 2.8 (s). ν/cm^{-1} ($CHCl_3$) (C–O–C–) 1100, (P=O) 1250, (CH_2-CH_2) 2850. Elemental analysis. Found: C, 58.17; H, 10.07. Calc. for $C_{126}H_{253}O_{51}P_1Na$: C, 57.38, 9.7%. TLC was carried out on silica gel 60 F254 precoated plates (Merck, Darmstadt, Germany) using a solvent system composed of chloroform–methanol–water 74:25:4 (v/v/v) as eluent. The R_f of DHP-PEG^{2000} **6** was 0.7, compared to 0.75 and 0.4, for PEG and dihexadecyl phosphatidic acid (DHP-PEG^{2000}), respectively. The spots were detected by molybdenum blue spray reagent (Sigma, St. Louis, MO, USA) for phosphorus compounds, and iodine as general detecting agents.[16]

Liposome preparation

Small unilamellar liposomes (vesicles) (SUV) were prepared from egg-PC (EPC 2, Lipoid KG, Ludwigshafen, Germany) at 8 mM lipid concentration in 50 ml of HEPES (20 mM) in saline (130 mM) buffer pH = 7.2 using a high-pressure homogenizer Model Minlab type 8.30 H (APV Rannie, Albertslund, Denmark) according to a published procedure.[17] Two preparations were made: one composed of only egg-PC (egg-PC SUV) and the second, a mixture of egg-PC with 3 mol% of DHP-PEG^{2000} (egg-PC/DHP-PEG^{2000} SUV).

Quantification of bound water

Evaluation of bound water level was done by heat flow differential scanning calorimetry (DSC)[4] using a Mettler Thermal Analyzer model 4000. The ice–water fusion enthalpy was evaluated by the peak integral at around 0 °C. Scanning was conducted from −30 °C to 10 °C at the rate of 2 °C min^{-1}. The exact amount of water bound to PEG^{2000} and DHP-PEG^{2000} was calculated from the decrease in the heat fusion enthalpy (ΔH_{fu}) of the HEPES–saline solutions containing the analyte, compared to free water HEPES–saline:

$$\%\text{PEG bound water} = [100 \times (\Delta H_{fu\ HEPES\text{-}saline} - \Delta H_{fu\ PEG/HEPES\text{-}saline})]/\Delta H_{fu\ water}$$

The fusion enthalpy of water was calculated from a calibration curve in which various amounts of water were scanned. ΔH_{fu} of water was 320 J/g^{-1} and of HEPES–saline, 275 J/g^{-1} [linear regression values (r^2) were above 0.99, n = 4].

Oxidative stress by irradiation

SUV (200 μl; 8 mM lipids) of either egg-PC or egg-PC/DHP-PEG^{2000} was γ-irradiated with a dose of 9200 Gy by ^{137}Cs in a Gamma-Cell 220 (Atomic Energy of Canada Inc., Ottawa) at a dose rate of 8.5 Gy min^{-1} under atmospheric conditions.

Oxidative stress by 2,2′-azo(2-amidinopropane) dihydrochloride (AAPH)

SUV (200 ml; 8 mM lipids) of either egg-PC or egg-PC/DHP-PEG^{2000} was incubated in two different experiments with 20 mM AAPH at 37 °C for 24 and 72 h, respectively.

Stability of liposomes to storage in air

Liposomes with and without DHP-PEG^{2000} were stored for 6 months at 4 °C. The liposomes were assayed every 2 months for loss of PUFA as an index of oxidative damage. After 4 months storage at 4 °C, samples of the liposomes were stored at 37 °C for 4 d to accelerate degradation.

Determination of phospholipid acyl chain composition in liposomes

SUV (100 μl; 8 mM lipids) before and after exposure to oxidative stress was diluted with 900 μl of water. The lipids were extracted from the aqueous phase by adding 1 ml of ethanol and 1 ml of chloroform and vortexing for 1 min. Two phases were formed, and the chloroformic phase was collected and dried by a steam of nitrogen followed by 2 h of lyophilization to remove all traces of water. The residue was dissolved in 50 μl of toluene, 10 μl of methanol and 20 μl of the methanol esterification reagent Meth-Prep II (Alltech). The methyl esters formed were analysed by GC (using a Perkin-Elmer 1020 plus GC, with a Silar 10C Alltech chromatographic column using a temperature gradient of 5 °C min^{-1} from 140 to 240 °C.[16]

Characterization of γ-irradiated DHP-PEG^{2000} liposomes by ^{1}H NMR

Liposomes containing 3 mol% of DHP-PEG^{2000} were irradiated for 18 h using a total dose of 9200 Gy. Then the lipids and the DHP-PEG^{2000} were extracted from the aqueous phase by 1 ml of ethanol and 1 ml of chloroform. Two phases were formed. All the phosphorus was found in the chloroform-rich lower phase, and none in the aqueous upper phase, indicating that all remained lipid-associated. The phase was dried by a stream of nitrogen followed by 2 h of lyophilization. The residue was redissolved in CCl_3D, and its ^{1}H NMR spectra were recorded. In order to evaluate the level of PEG fragmentation induced by oxidation, we quantified by ^{1}H NMR the protons of PEG and choline moieties and calculated the PEG/choline ^{1}H NMR integration ratios before and after irradiation.

Lipid hydroperoxide accumulation assay

Lipid hydroperoxides were monitored using a sensitive spectroscopic method[18] which was modified to the micromolar range: 50 μl of liposome dispersion was dissolved in 1 ml of ethanol. Aqueous potassium iodine (50%; 50 μl) was added and the mixture was incubated for 30 min in the dark. Absorbance at 400 nm was measured.

AAPH adsorption on liposome surface

AAPH is a water-soluble positively charged molecule. As such, it may be adsorbed on negatively charged surfaces such as those of the DHP-PEG^{2000}-containing liposomes. This was evaluated from AAPH-induced changes in liposome electrical surface potential calculated from the shift in apparent pK_a of HC (Molecular Probes, Eugene, OR, USA). Unilamellar liposomes of egg-PC and of egg-PC with 3 mol% DPH-PEG^{2000} (lipid concentration of 125 mM) containing the fluorescent HC probe (probe:lipid 1:200, mol:mol) were prepared by medium pressure extrusion through polycarbonate filters of 100 nm pore size.[17] The fluorescence excitation spectrum (300–400 nm range), using constant emission at 450 nm, was recorded at different bulk pH values (pH range 7.0–13.0), from which the apparent pK_a of HC was determined. The shift in pK_a in the presence and absence of AAPH was used to calculate electrical surface potential.[19]

Acknowledgements

This research was supported by a grant from the Alex Grass Center for Drug Design and Synthesis. We thank Dr Akiva Vexler, Sharett Institute of Oncology and Ms Anat Alon, Department of Pharmacy, for technical assistance; Professor Eli Breuer for reviewing the chemical section; and Mr Sigmund Geller for help in editing the manuscript.

References

1 D. Chapman, R. M. Williams and B. D. Ladbrooke, *Chem. Phys. Lipids*, 967, **1**, 445.

2 D. W. R. Gruen, S. Marcèlja and V. A. Paresgian, in *Cell Surface Dynamics, Concepts and Models*, eds. A. S. Perelson, C. Delisi and F. W. Wiegel, Marcel Dekker, New York, 1984, pp. 59–91.
3 L. R. C. Barclay and K. U. Ingold, *J. Am. Chem. Soc.*, 1981, **103**, 6478.
4 Y. Barenholz, E. Freire, T. E. Thompson, M. C. Correa-Freire, D. Bach and I. R. Millar, *Biochemistry*, 1983, **22**, 3497.
5 *Stealth Liposomes*, eds. D. Lasic and F. Martin, CRC Press, Boca Raton, FL, 1995.
6 *Membrane Fashion*, eds. T. B. Lawrence, H. Sek-Wen, J. Wilschut and D. Hoekstra, Marcel Dekker, New York, 1991, pp. 231–253.
7 S. V. Jovanovic, I. Jankovic and L. Josimovic, *J. Am. Chem. Soc.*, 1992. **114**, 9018.
8 G. R. Buettner, *Arch. Biol. Biophys.*, 1993, **300**, 535.
9 J. Israelachivili, *Intermolecular and Surface Forces*, Academic Press, 2nd edn., pp. 367–370.
10 *Free Radicals in Biology and Medicine*, eds. B. Halliwell and J. M. C. Gutteridge, Clarendon Press, Oxford, 1989, 2nd edn., pp. 22–81
11 G. Stark, *Biochim. Biophys. Acta*, 1991, **1071**, 103
12 M. M. Dooley, N. Sano, H. Kaeashima and T. Nakamura, *Free Radical Biol. Med.*, 1990, **9**, 199.
13 N. J. Zuidam, S. S. L. Lee and D. S. A. Crommelin, *Pharm. Res.*, 1995, **12**, 1761.
14 I. Lindh and J. Stawinski, *J. Org. Chem*. 1989, **54**, 1338.
15 M. Kates, T. H. Chan and N. Z. Stanacev, *Biochemistry*, 1963, **2**, 394.
16 Y. Barenholz and S. Amselem, in *Liposome Technology*, ed. G. Gregoriadis, CRC Press, Boca Raton, FL, 1993, 2nd edn., pp. 501–525.
17 G. Haran, R. Cohen, L. K. Bar and Y. Barenholz, *Biochim. Biophys. Acta*, 1993, **1151**, 201.
18 *The Determination of Organic Peroxides*, eds. R. M. Johnson and I. W. Siddiqi, Pergamon Press, New York, 1966, pp. 50–52.
19 R. Pal, W. A. Petri, Jr., Y Barenholz and R. R. Wagner, *Biochim. Biophys. Acta*, 1983, **792**, 185.
20 A. Samuni and Y. Barenholz, *Free Radical Biol. Med.*, 1996, in the press.

Paper 6/01955B
Received 20*th March* 1996
Accepted 22*nd October* 1996