Gamma-Irradiation of Non-Frozen, Frozen, and Freeze-Dried Liposomes

Nicolaas J. Zuidam,[1,2] Stephen S. L. Lee,[1] and Daan J. A. Crommelin[1]

Received November 11, 1994; accepted February 10, 1995

Purpose. The aim of this work was to investigate the possibilities and limitations of gamma-irradiation as a sterilisation method for non-frozen, frozen, and freeze-dried liposomes.

Methods. Liposomes with an average size of 0.2 μm were irradiated with doses up to about 5×10^4 Gy in a nitrogen atmosphere.

Results. Phospholipids in dipalmitoylphosphatidycholine/dipalmitoylphosphatidylglycerol (DPPC/DPPG) 10/1-liposomes and egg phosphatidylcholine/egg phosphatidylglycerol (EPC/EPG) 10/1-liposomes in 10 mM phosphate buffer (pH 7.4) without trehalose degraded considerably upon gamma-irradiation. Irradiation damage was reduced in the presence of 10% trehalose added as a cryoprotectant, but trehalose reacted with species induced by gamma-irradiation as demonstrated by large decreases in pH. Both pH decrease and oxidative damage of EPC/EPG 10/1-liposomes were strongly dependent on the physical state during irradiation (non-frozen, frozen or freeze-dried). No changes in liposomal size were found upon gamma-irradiation, and hardly any change was seen in bilayer rigidity. Differences in the gel-to-liquid phase transition of DPPC/DPPG 10/1-liposome dispersions before and after gamma-irradiation were small in the presence of 10% trehalose, but larger in the absence of trehalose.

Conclusion. The degradation of trehalose limits the use of freezing or freeze-drying liposome dispersions as a way to minimise irradiation damage.

KEY WORDS: liposomes; sterilisation; chemical and physical stability; gamma-irradiation; freeze-drying; differential scanning calorimetry (DSC).

INTRODUCTION

A large number of publications appeared over the years demonstrating damage to liposome dispersions upon gamma-irradiation, even for liposomes composed of saturated phospholipids under an inert atmosphere (see (1,2), references in these articles, (3,4)). Therefore, exposure to gamma-irradiation is thought to be unsuitable to sterilise liposomes to be used as drug carriers (5). However, several publications appeared over the years suggesting that sterilisation of liposomes by gamma-irradiation was not as detrimental to the liposome formulations as generally assumed (6-9). These articles reported that liposomes were sterilised by exposure to doses of 12.5×10^3 Gy, 15 or 20×10^3 Gy, 15×10^3 Gy and 15×10^3 Gy, respectively. Upon gamma-irradiation no effect on liposome-entrapped enzyme activity (6), nor modification in therapeutic efficacy in mice of egg phosphatidylcholine/cholesterol/stearylamine 4/3/1-liposomes containing a water-insoluble cytostatic agent, NSC 251635 (7), nor modification of the elution profile of the same dispersion from a Sepharose Cl-4B column were observed (7). No information was provided about the atmosphere during the gamma-irradiation of the liposomes. NCS 251635 containing liposomes, sterilised by gamma-irradiation, were even administered intravenously to terminal patients without serious side effects (8,9). However, other liposome characteristics than those mentioned above were not investigated after gamma-irradiation and, therefore, any questions are still left.

Some Russian articles reported that the most effective way to protect drug solutions or liposome dispersions from irradiation damage is freezing (10,11). This method is called cryoradiation. With cryoradiation the capacity to inactivate viruses or microorganisms upon exposure to a dose of 25×10^3 Gy is maintained. By freezing, the damaging effect by the indirect irradiation action is suppressed. The damage by the indirect action is not caused by direct 'hits' on the bilayer structure by the photons, but it is caused indirectly: by reactive agents outside the bilayer that are generated by gamma-irradiation. Not only the temperature in the frozen state, but also the structure of the frozen media affects the stability of drugs. Upon slow freezing, the original solution will form segregated micro grains. Then, indirect action of radiation will only take place at the boundaries as demonstrated by electron spin resonance (ESR) measurements. The bigger a grain, the less the indirect action. Kapanin *et al.* found no increase in oxidation and no decrease in insulin content after gamma-irradiation of liposomes containing insulin while freezing at −196°C and using 10% chloride as a cryoprotectant (10).

The aim of the present study was to investigate in more detail the effect of exposure of liposomes composed of saturated and unsaturated phospholipids in a nitrogen atmosphere to gamma-irradiation in the form of a non-frozen or frozen dispersion, or of a freeze-dried product. Gamma-irradiation of liposomes in a frozen or freeze-dried state was performed to investigate if the indirect action of gamma-irradiation could be suppressed under those conditions. Phosphate buffer was used, because no change in pH was found after gamma-irradiation of this buffer (4). To reduce physical changes of the liposomes by the freezing or freeze-drying procedure, trehalose was used as a cryoprotectant in these experiments. To find out the effect of trehalose on the irradiation damage of non-frozen liposome dispersions, these dispersions were irradiated with and without this cryoprotectant. To allow for monitoring size changes after gamma-irradiation treatment, liposomes were sized during their preparation process and had an average size of about 0.2 μm before the gamma-irradiation. Chemical degradation of the liposome dispersions was monitored as a function of the irradiated dose by measuring the pH, by monitoring the extent of lipid oxidation of the unsaturated phospholipids (oxidation-index) and the concentration of phospholipids by HPLC. Physical changes induced by gamma-irradiation were assessed by determining the size of the liposomes, the

[1] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), participant in Groningen Utrecht Institute for Drug Exploration (GUIDE), Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands.

[2] To whom correspondence should be addressed at Department of Pharmaceutics, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands.

0724-8741/95/1100-1761$07.50/0

gel-to-liquid state transition of the liposomes composed of saturated phospholipids by differential scanning calorimetry and the bilayer rigidity by fluorescence anisotropy measurements of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH).

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were gifts from Nattermann Phospholipid GmbH (Cologne, FRG). Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were gifts from Lipoid K.G. (Ludwigshafen, FRG). Trehalose was supplied by Sigma Chemicals (St. Louis, Missouri, USA). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Janssen Chimica (Beerse, Belgium). These and all other chemicals were of analytical grade. The water was double-distilled before use.

Preparation of Liposomes

Liposomes were prepared by the "film" method. Appropriate mixtures of the phospholipids were dissolved in chloroform/methanol (1:1) in a round-bottom flask. The organic solvent was removed under vacuum by rotary evaporation. The thin film obtained was dried for at least three hours under reduced pressure. Then the film was hydrated with 10 mM phosphate buffer (pH 7.4) with or without 10% trehalose. The liposome dispersions were extruded with an extrusion system (Sartorius, Göttingen, FRG) through 0.6 μm and three times through 0.2 μm pore size filters, respectively (Nuclepore, Costar Corporation, Cambridge, Massachusetts, USA). The pH of the dispersion was measured before and after extrusion and adjusted, if necessary. The final phospholipid concentration was about 22 mM. Nitrogen was bubbled through a part of the batch in a nitrogen filled cabinet for at least 30 minutes to remove oxygen. Two ml aliquots of the liposome dispersions were filled into 10 ml vials. The vials were closed with viton-stoppers (Rubber B.V., Hilversum, The Netherlands). According to the manufacturer, viton is very stable and is impermeable for gasses. A part of these vials with the liposome dispersions was frozen by exposure to dry-ice (−79°C). Another part of the batch was freeze-dried in a Leybold GT4 pilot-production freeze-dryer. Two ml aliquots of the dispersions were freeze-dried in 10 ml vials for three days at −40°C and at a pressure of 10 Pa. To minimise the water content a secondary drying phase followed at 20°C for 11 hours at a pressure of 4 Pa. Butyl rubber stoppers had to be used to close the vials in the freeze-dryer under low pressure. These stoppers were replaced by viton-stoppers in the nitrogen filled cabinet. The residual moisture contents were determined with Karl-Fisher titration performed with a Mitsubishi moisturemeter model CA-05 (Tokyo, Japan). The residual water content of the freeze-dried dispersions was 0.6 ± 0.1% of the weight of the freeze-dried cake.

Irradiation

The samples were irradiated with a ^{60}Co source by Gammaster B.V. (Ede, The Netherlands) in boxes at ambient temperature. The liposome dispersions in 10 mM phosphate without trehalose were irradiated with a dose rate of 7.8×10^3 Gy/h when exposed to a dose of 1.3×10^4 Gy and with a dose rate of 4.4×10^3 Gy/h when the samples were exposed to a dose of 2.8×10^4 Gy or 5.8×10^4 Gy. The liposomes in 10 mM phosphate and 10% trehalose were irradiated with a dose rate of 1.1×10^3 Gy/h. These samples were exposed to a dose of 1.3×10^4, 2.9×10^4 or 5.0×10^4 Gy. The absorbed doses reported here are minimum doses. The dosimeters used consisted of red perspex which gave readings within 5% (as stated by Gammaster, see also (12)). Earlier it was found that differences in the high dose rates used in this study did not affect the irradiation damage (4).

Differential Scanning Calorimetry (DSC)

Liposomes were concentrated by ultracentrifugation at 200,000 *g* for 30 minutes. The pellet was put into an aluminum pan. As a reference an empty aluminum pan was used. Calorimetric scans from 25 to 60°C were performed on a Netzsch DSC 200 low temperature DSC (Netzsch-Gerätebau, Selb, FRG). The scanning rate was 2°C/minute. The amount of phospholipids in the pan was determined by a phosphate determination (13) in the lower phase of a Bligh & Dyer-extract (14) of the content of the pan.

Fluorescence Anisotropy

To gain information about the bilayer rigidity of the liposomes the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in liposomes was measured. 5 μl of 2×10^{-4} M DPH, dissolved in tetrahydrofuran, was added to 25, 50 and 75 μl of the liposome dispersion diluted in buffer (total volume 3 ml). This mixture was stabilised one hour in the dark at ambient temperature in the case of EPC/EPG 10/1-liposomes or, in the case of DPPC/DPPG-liposomes, at 60°C. The anisotropy measurements were performed on a LS50 luminescence spectrometer (Perkin Elmer Ltd., Norwalk, Connecticut, USA) at 20 ± 1°C, using an excitation wavelength of 365 nm (band width 5 nm) and an emission wavelength of 430 nm (band width 5 nm). Anisotropy values (r) were computed after correction for optical and electronic differences in the parallel and perpendicular channels (G-factor). The corrected r of a dispersion was obtained after extrapolation of the values for r to a liposome concentration of zero as proposed by Litman and Barenholz (15).

Analytical Methods

Phospholipids were analysed by HPLC as described earlier (16). Samples for the HPLC analysis were prepared by the Bligh and Dyer-extraction (14). The phospholipids were collected in the chloroform phase. After dilution of the chloroform phase in methanol, 100 μl was directly injected into the column. The HPLC system consisted of a type 400 solvent delivery system (Kratos, Ramsey, New Jersey, USA), a Kontron sampler MSI 660 (Kontron AG, Zürich, Switzerland) and a Waters 410 RI detector (Waters Association, Milford, Massachusetts, USA). Chromatograms were collected and analysed with a computerised data system (WOW, Thermo Separation Products, Riviera Beach, Flor-

ida, USA). The separation of the phospholipids was carried out on a Zorbax aminophase column (25 cm × 4.6 mm, I.D., 5 μm particle size, Du Pont Company, Wilmington, DE, USA) at 35°C. An Adsorbosphere NH2 5μ-guard column (Alltech Association, Deerfield, Illinois, USA) was connected before the Zorbax aminophase column. The mobile phase consisted of acetonitrile/methanol/5 mM ammonium dihydrogen phosphate solution pH 4.8 (64/26/5, v/v). The flow rate was 1.5 ml/minute.

Oxidation of the egg phospholipids was determined by monitoring the formation of conjugated dienes (17). After a Bligh and Dyer-extraction (14) of a liposome dispersion, part of the lower phase was evaporated and dissolved in pure ethanol. The oxidation-index was estimated as the absorption ratio at 233 nm/215 nm after correction for the background measured at 300 nm (oxidation-index = $(A_{233nm} - A_{300nm})/(A_{215nm} - A_{300nm})$). The measurements were performed on a double beam spectrophotometer (Lambda 2, Perkin-Elmer Corporation, Norwalk, Connecticut, USA).

The Z-average particle size and polydispersity index (p.d.) were determined by dynamic light scattering (DLS) at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC Corp., Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd., Malvern, UK). The p.d. is a measure for the width of the particle size distribution and ranges from 0.0 for an entirely homogeneous dispersion up to 1.0 for a dispersion with a completely heterogeneous size distribution. When 10% trehalose was present in the 10 mM phosphate buffer, the value for the refractive index used was 1.348 as determined by a refractometer. The value for the viscosity of the 10 mM phosphate buffer with 10% trehalose was 1.078 $g.m.^{-1}.s^{-1}$ as determined by DLS measurements of standard latex particles according to the method described by De Smidt and Crommelin (18). In the absence of trehalose, the values for refractive index and viscosity of pure water were used.

Statistics

Significance tests on a mean were performed by using the Student t-test assuming equal variances and using a value for α of 0.05 (two sided).

RESULTS

Charged and sized liposomes composed of phospholipids with different degrees of saturation of their acyl chains were made: DPPC/DPPG 10/1 and EPC/EPG 10/1. These liposomes were irradiated as non-frozen or frozen dispersions or as a freeze-dried product. After gamma-irradiation the frozen liposomes were thawed at ambient temperature. The freeze-dried liposomes were reconstituted by addition of water at ambient temperature. The amount of water necessary for rehydration was derived from mass measurements of the samples before and after the freeze-drying process.

The pH values of the liposome dispersions and buffers before and after gamma-irradiation were compared. The change in pH of DPPC/DPPG 10/1-liposomes upon gamma-irradiation is shown in Fig. 1. The changes in pH of EPC/EPG 10/1-liposomes and buffers were not significantly different. Before gamma-irradiation all liposome dispersions and buffers had an initial pH of about 7.4 ($\Delta pH \leq 0.1$). The

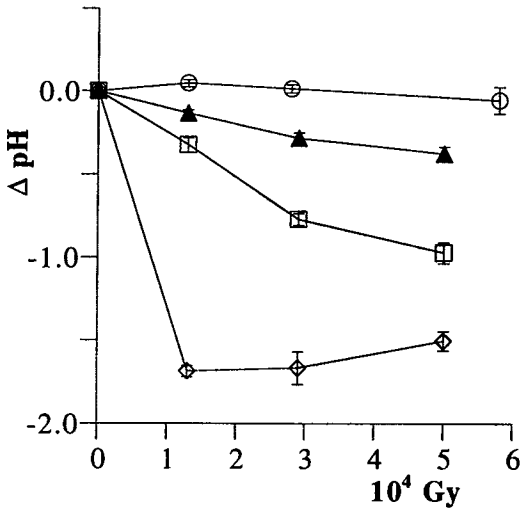

Fig. 1. The change in pH of DPPC/DPPG 10/1-liposomes upon gamma-irradiation in different physical states. The initial pH was 7.4. The changes in pH of EPC/EPG 10/1-liposomes and buffers upon gamma-irradiation were not significantly different. Vertical bars denote S.D. for three determinations. When no bars are shown, S.D. fell within symbol dimensions. ○: non-frozen without trehalose; ▲: freeze-dried with trehalose; □: non-frozen with trehalose; ◇: frozen with trehalose.

pH of liposome dispersions and buffers without trehalose was stable upon gamma-irradiation. All liposome dispersions and buffers containing trehalose showed a drop upon gamma-irradiation. Interestingly, the decrease in pH depended on the physical state of the liposome dispersions during the irradiation treatment. The data also show that the presence of liposomes did not affect the pH drop.

Oxidation of the unsaturated EPC/EPG 10/1-liposomes was monitored by measuring the oxidation-index (see Fig. 2). An increase was observed when the non-frozen, frozen,

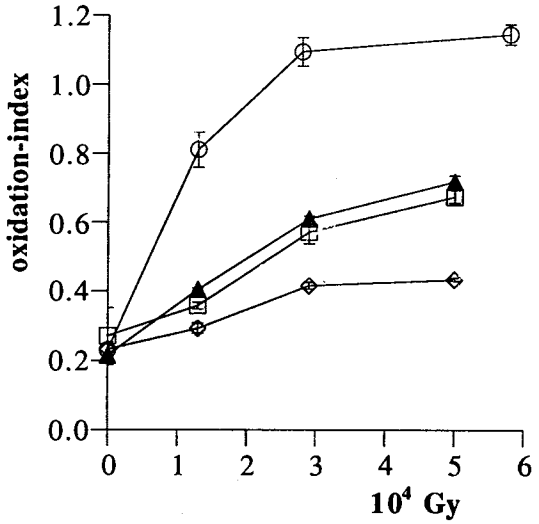

Fig. 2. The oxidation-index of EPC/EPG 10/1-liposomes with or without trehalose upon gamma-irradiation in different physical states. Vertical bars indicate S.D. for three determinations. When no bars are shown, S.D. fell within symbol dimensions. For symbols see Fig. 1.

or freeze-dried liposome dispersions were irradiated. The presence of trehalose suppressed the increase in oxidation-index of the EPC/EPG 10/1-liposomes upon gamma-irradiation. Interestingly, the frozen dispersion showed less increase in oxidation-index than the non-frozen or freeze-dried liposomes.

In Fig. 3A-D the results of HPLC analysis of the phospholipids of the liposome dispersions are shown. Upon gamma-irradiation the concentration of phospholipids in non-frozen, frozen and freeze-dried liposome dispersions in 10 mM phosphate buffer and 10% trehalose did not decrease significantly, except DPPG in DPPC/DPPG 10/1-liposomes. However, the concentration of phospholipids in non-frozen liposome dispersions showed a pronounced decrease in 10 mM phosphate buffer without trehalose. In a previous article (4) linearity was found when the logarithmic values of the remaining phospholipid concentration were plotted against the irradiation dose. In the present study, this observation was confirmed for the liposome dispersions without trehalose; the slopes for the degradation of DPPC and DPPG in 22 mM DPPC/DPPG 10/1-liposomes were $4.1 \times 10^{-6} \pm 0.5 \times 10^{-6}$ Gy^{-1} and $26 \times 10^{-6} \pm 2 \times 10^{-6}$ Gy^{-1} and the slopes for the degradation of EPC and EPG in 22 mM EPC/EPG 10/1-liposomes were $8 \times 10^{-6} \pm 1 \times 10^{-6}$ Gy^{-1} and $9 \times 10^{-6} \pm 2 \times 10^{-6}$ Gy^{-1}. Because hardly any degradation of the liposomal phospholipids occurred in the presence of trehalose, the values for these slopes are not shown here. Hardly any increase (1-2 mol% of the phosphatidylcholine) was observed in the lysophosphatidylcholine concentration, even in cases where considerable degradation of PC was observed.

To find out whether gamma-irradiation had affected the physical properties of the liposomes, the size of the liposomes, the melting properties of the DPPC/DPPG 10/1-liposomes and their bilayer rigidity were estimated before and after gamma-irradiation.

No changes in liposome size were detected after a freezing-thawing cycle or freeze-drying rehydration cycle of liposomes in 10 mM phosphate buffer and 10% trehalose. Irradiation had no effect on the liposome size either. All dispersions had average sizes of around 0.2 μm and had a p.d. of about 0.1 (indicating rather narrow size distributions; results not shown).

Upon gamma-irradiation changes in the gel-to-liquid state transition of DPPC/DPPG 10/1-liposomes were measured by DSC (see Fig. 4A-D). The summarised melting characteristics and abbreviations are shown in Table I. Interestingly, a change in the melting properties of DPPC/DPPG 10/1-liposomes was observed upon freeze-drying (see Fig. 4B). Both irradiated and non-irradiated liposomes showed this change. The frozen liposomes did not show this phenomenon (see Fig. 4D). Apparently, the bilayer structure

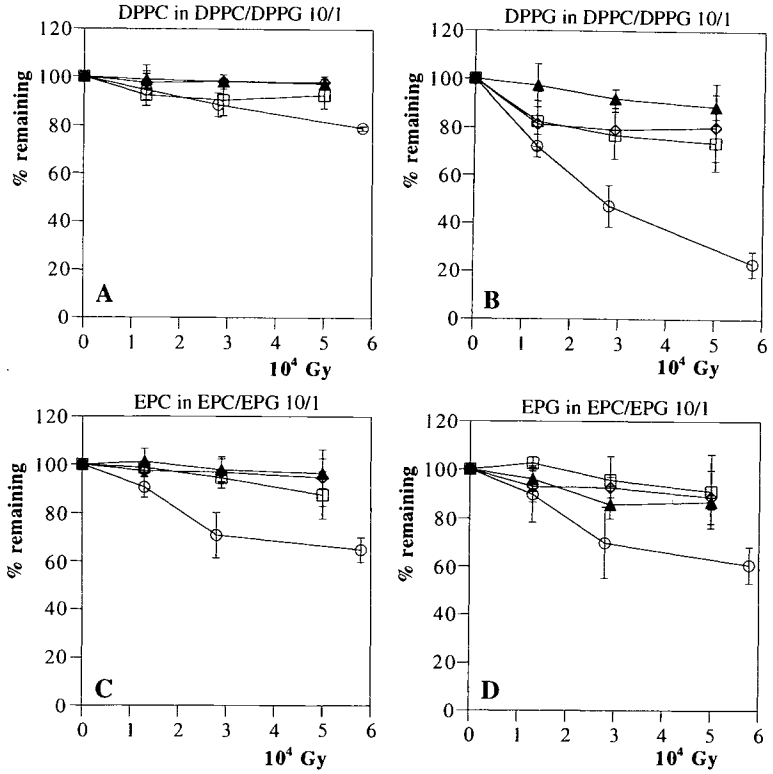

Fig. 3. Results of the HPLC analysis of gamma-irradiated DPPC/DPPG 10/1- and EPC/EPG 10/1-liposomes in 10 mM phosphate buffer (pH 7.4) with or without 10% trehalose and in different physical states. Vertical bars denote S.D. for three determinations. When no bars are shown, S.D. fell within symbol dimensions. For symbols see Fig. 1. A: DPPC in DPPC/DPPG 10/1-liposomes; B: DPPG in DPPC/DPPG 10/1-liposomes; C: EPC in EPC/EPG 10/1-liposomes; D: EPG in EPC/EPG 10/1-liposomes.

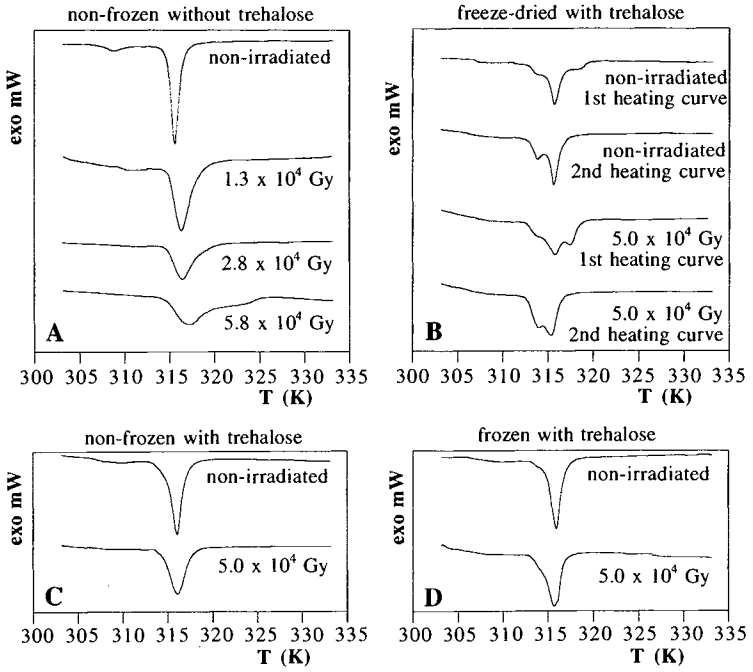

Fig. 4. Typical examples of DSC-scans of DPPC/DPPG 10/1-liposome dispersions before and after irradiation in different physical states at a dose as indicated in the figure. See also Table I for description of the melting characteristics. A: irradiation as a non-frozen liposome dispersion without trehalose. B: irradiation as a freeze-dried liposomal product with trehalose: C: irradiation as a non-frozen liposome dispersion with trehalose; D: irradiation as a frozen liposome dispersion with trehalose.

was affected by the freeze-drying process in the presence of trehalose. The scans changed when the same samples were measured for a second time (see Fig. 4B), indicating that (at least part of) the phenomenon had a non-permanent character. No differences between a second and a third heating scan were observed.

The pre-transitions of the dispersions with trehalose were hardly detectable by the method used (see Fig. 4B-D). Therefore, the characteristics of these pre-transitions are not mentioned in Table I. Upon gamma-irradiation, the pre-transition of the DPPC/DPPG 10/1-liposomes in 10 mM phosphate without trehalose disappeared (see Fig. 4A and Table I). No change in T_m of the pre-transitions (36 ± 1°C), if detectable, was observed. The main phase transition of all DPPC/DPPG 10/1-liposomes broadened upon gamma-irradiation; the $\Delta T_{1/2}$ became larger. A change in ΔH_m was not observed. Also T_{on} and the T_m hardly changed upon gamma-irradiation. After exposure of DPPC/DPPG 10/1-liposomes in phosphate buffer without trehalose to a dose of 5.8×10^4 Gy, a shoulder at the right hand side of the main transition was observed (see Fig. 4A). The broadening of the peaks was less pronounced after gamma-irradiation of the liposomes in the presence of trehalose (see Fig. 4B-D and Table I).

To monitor the consequences of gamma-irradiation on the rigidity of the phospholipid bilayers, the fluorescent anisotropy of a lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was measured. The anisotropy values were 0.35 ± 0.01 for all DPPC/DPPG 10/1-liposomes and 0.12 ± 0.01 for all EPC/EPG-liposomes. The anisotropy values of the liposomes in 10 mM phosphate buffer with 10% trehalose remained unchanged after a freezing-thawing cycle or freeze-drying-rehydration cycle (results not shown). Also no change in anisotropy values could be found upon gamma-irradiation of these liposomes (results not shown). However, a small increase was observed upon gamma-irradiation of the EPC/EPG 10/1-liposomes in 10 mM phosphate buffer without trehalose. Then the anisotropy values after exposure of a dose of 0, 1.3×10^4, 2.8×10^4 or 5.8×10^4 Gy were 0.132 ± 0.008, 0.131 ± 0.007, 0.147 ± 0.013 or 0.152 ± 0.006, respectively. A t-test showed that a statistically significant difference was only observed between the first or second anisotropy value and the last anisotropy value ($p < 0.05$).

DISCUSSION

When non-frozen dispersions of liposomes are gamma-irradiated, water molecules will be the main source of free radicals. The main primary products of water radiolysis are OH·, H· and the hydrated electron e_{aq}. The fourth product, H_2O^+, reacts fast to OH· and to H_3O^+ (19). This indirect irradiation effect is more damaging for bilayers than the direct irradiation effect (where irradiation is absorbed directly by the lipid molecules), because free radicals generated by radiolysis of water have unlimited access to the membrane (19). In this study, an attempt was made to minimise the indirect irradiation effect by reducing the mobility of water soluble reactive species (induced by gamma-irradiation) by freezing or by removing water by freeze-drying.

In Fig. 1, the change in pH upon gamma-irradiation of DPPC/DPPG 10/1-liposomes is shown. The results upon gamma-irradiation of EPC/EPG 10/1-liposomes and buffers

Table I. The Melting Characteristics of DPPC/DPPG 10/1-liposomes Before and After Gamma-Irradiation[a]

State during irradiation	With trehalose	Dose ($\times 10^4$ Gy)	ΔH_m (kJ/mol P)	ΔH_p (kJ/mol P)	T_{on} (°C)	T_m (°C)	$\Delta T_{1/2}$ (°C)
non-frozen	no	0	34 ± 3	3.2 ± 0.2	41.6 ± 0.1	42.6 ± 0.3	1.1 ± 0.2
non-frozen	no	1.3	34 ± 3	0.5 ± 0.8	41.8 ± 0.3	43.1 ± 0.2	1.6 ± 0.3
non-frozen	no	2.8	33 ± 1	nd	41.8 ± 0.3	43.5 ± 0.3	2.1 ± 0.3
non-frozen	no	5.8	33 ± 3	nd	41.7 ± 0.3	44.2 ± 0.7	4.0 ± 1.6
non-frozen	yes	0	32 ± 5	—	41.7 ± 0.2	43.0 ± 0.3	1.3 ± 0.1
non-frozen	yes	1.3	32 ± 1	—	41.5 ± 0.3	43.0 ± 0.3	1.5 ± 0.3
non-frozen	yes	2.9	30 ± 4	—	41.6 ± 0.1	43.1 ± 0.1	1.2 ± 0.5
non-frozen	yes	5.0	33 ± 1	—	40.9 ± 0.3	43.0 ± 0.1	2.1 ± 0.2
frozen	yes	0	33 ± 1	—	41.6 ± 0.1	42.8 ± 0.1	1.3 ± 0.1
frozen	yes	1.3	33 ± 2	—	41.5 ± 0.1	43.0 ± 0.1	1.5 ± 0.1
frozen	yes	2.9	35 ± 2	—	41.3 ± 0.1	42.9 ± 0.2	1.6 ± 0.1
frozen	yes	5.0	32 ± 2	—	41.2 ± 0.2	42.7 ± 0.3	1.5 ± 0.3
freeze-dried	yes	0	32 ± 3	—	41.5 ± 0.1	42.5 ± 0.1	1.1 ± 0.1
freeze-dried	yes	1.3	34 ± 2	—	41.3 ± 0.1	42.6 ± 0.1	1.3 ± 0.1
freeze-dried	yes	2.9	33 ± 5	—	41.1 ± 0.2	42.9 ± 0.1	1.9 ± 0.1
freeze-dried	yes	5.0	36 ± 2	—	40.8 ± 0.5	42.9 ± 0.3	2.5 ± 0.8

[a] Data represent the mean of triplicate-experiments ± S.D. ΔH_m is the enthalpy of the $P_\beta' \rightarrow L_\alpha$ transition, the so-called gel-to-liquid phase transition or main phase transition; ΔH_p is the enthalpy of the $L_\beta' \rightarrow P_\beta'$ transition, the so-called pre-transition; T_{on} is the temperature at the onset of the main phase transition; T_m is the temperature at the minimum of the main phase transition; $\Delta T_{1/2}$ is the width of the main-transition at half height; P is phosphate; nd means not detectable by the method used; — is not evaluable, because the pre-transition could not always be clearly defined under the experimental conditions (see also text).

were not statistically different. The pH of liposome dispersions and buffers without trehalose in a nitrogen atmosphere was stable upon gamma-irradiation ($\Delta pH < 0.1$). Previously, it was found that upon gamma-irradiation of liposome dispersions without trehalose in an air atmosphere the pH decreased (around 0.5 units after a dose of about 5×10^4 Gy, depending on the type of liposomes and degree of degradation) (4). Apparently, the drop in pH observed in that study was caused by oxygen-depending reaction(s). Trehalose containing samples showed a decrease in pH upon gamma-irradiation (see Fig. 1). Interestingly, the change in pH also depended on the physical state of the liposome dispersions and buffer during the irradiation treatment: gamma-irradiation of the frozen liposome dispersions and buffer resulted in a large drop in pH compared to gamma-irradiation of the freeze-dried products. The presence of liposomes did not affect the pH drop, indicating that the proton formation should be ascribed to degradation of the cryoprotectant trehalose.

Although oxygen was removed from the vials and replaced by nitrogen the liposomes composed of unsaturated phospholipids were oxidized after gamma-irradiation (see Fig. 2). The presence of trehalose suppressed this increase in oxidation-index. Interestingly, the frozen dispersion showed less increase in oxidation-index than the non-frozen or freeze-dried liposomes. A study demonstrating no increase in oxidation at all upon gamma-irradiation of liposomes composed of unsaturated phospholipids in the absence of oxygen has never been published. Just recently, Memoli *et al.* showed that even under nitrogen a minor increase in oxidation-index could be found upon probe-sonication of EPC-liposomes (20). Apparently, removing all traces of oxygen is very difficult. Possible approaches failed to further reduce oxidation damage upon gamma-irradiation of non-frozen dispersions (without trehalose) in an inert atmosphere by addition of antioxidants to the water (sodium metabisulfiet) and/or bilayer phase (butyl-hydroxytoluene, BHT) and/or the use of complexing agents for heavy metals (EDTA) (results not shown). Because safety data are lacking on the use of oxidized lipids in liposomes, gamma-irradiation of liposome dispersions seems primarily to be an option for sterilisation of liposomes composed of saturated phospholipids. If an effective and safe antioxidant protecting liposomes containing unsaturated phospholipids could be identified, then unsaturated lipids might become acceptable candidates as building stones for liposomes that have to be gamma-sterilised.

The HPLC analysis of phospholipids showed that trehalose interfered with the reaction between the species induced by gamma-irradiation and the liposomal phospholipids. Upon gamma-irradiation phospholipids of liposomes in 10 mM phosphate without trehalose were more degraded than phospholipids in dispersions containing trehalose (see Fig. 3. As observed before (4), DPPG in the DPPC/DPPG 10/1-liposomes degraded easier than DPPC (compare Fig. 3A and Fig. 3B). In the present study HPLC analysis of phospholipids demonstrated also a decrease in the concentration of both EPC and EPG in EPC/EPG 10/1-liposomes in 10 mM phosphate without trehalose. However, unlike the saturated phospholipids no difference in degree of degradation of phospholipids between EPC (neutral) and EPG (negatively charged) was found. The ranking order for the degree of degraded phospholipid in the liposome compositions used in 10 mM phosphate buffer without trehalose showed the following trend: DPPC < EPC = EPG < DPPG. Apparently, both the nature of the headgroup of the phospholipids and the degree of saturation of the fatty acids influenced the degradation of phospholipids upon gamma-irradiation.

As found before (4) the size of the liposomes was not affected by gamma-irradiation. Upon gamma-irradiation the anisotropy values of DPH in the liposomal bilayers hardly

changed, indicating that the rigidity of the bilayers was not affected. These results are in agreement with earlier observations (4). Only upon gamma-irradiation of EPC/EPG 10/1-liposomes in 10 mM phosphate buffer without trehalose a small increase was found. These liposomes were also more oxidized than the other liposomes used in this study (see Fig. 2). It has been reported before that lipid peroxidation increases the bilayer rigidity (2,21), especially the region near the double bonds of the phospholipids (21).

A change in the gel-to-liquid phase transition of the DPPC/DPPG 10/1-liposomes was observed upon freeze-drying and reconstitution (no gamma-irradiation, see Fig. 4B). This phenomenon has never been reported before. No such change was observed with liposomes after a freezing-thawing cycle. Viera *et al.* also found differences in physiochemical properties (permeability, effect of bilayer interacting compounds) after freeze-drying and rehydration of multilamellar liposomes composed of DPPC mixed with different amounts of trehalose (22). However, in contrast to the results in the present study they reported that DSC scans of reconstituted freeze-dried liposomes and non-freeze-dried liposomes did not differ. Maybe, this discrepancy can be ascribed to differences in liposome size and morphology. Further investigations have to establish a possible relationship between these changes in thermotropic behaviour and changes in marker retention and marker permeability (compared to non-freeze-dried liposomes) after reconstitution of freeze-dried liposomes. It was found before that a substantial percentage of carboxyfluorescein leaked out upon a freeze-drying-rehydration cycle for liposomes composed of hydrogenated soybean phosphatidylcholine and dicetylphosphate in a 10/1 molar ratio in the presence of a cryoprotectant (23,24). Leakage depended both on the residual water content as well on the particle size.

Changes in the gel-to-liquid phase transition of DPPC/DPPG 10/1-liposomes in 10 mM phosphate buffer and 10% trehalose were observed upon gamma-irradiation of non-frozen, frozen or freeze-dried liposomes (see Fig. 4 and Table I). Only some broadening of the main phase transition was found. Much more pronounced changes in melting properties were found after gamma-irradiation of DPPC/DPPG 10/1-liposomes in 10 mM phosphate buffer without trehalose (see Fig. 4 and Table I), which might be ascribed to the presence of degradation products such as dipalmitoylphosphatidic acid (DPPA) (4,25). Similar scans were obtained upon gamma-irradiation of DPPC/DPPG 10/1-liposomes in 10 mM phosphate buffer and 0.13 M NaCl in an air atmosphere (4). Therefore, the irradiation damage of liposomes composed of saturated phospholipids is not influenced by sodium chloride and atmosphere (air/nitrogen).

The DSC measurements demonstrated that the presence of trehalose reduces the irradiation damage. As discussed before (4), the results of the HPLC analysis of phospholipids and the results of the DSC measurements correlated qualitatively in this study.

This study was performed to find out if gamma-irradiation is a suitable method to sterilise liposomes. Phospholipids, encapsulated drugs (if present) and additives have to remain stable upon sterilisation. As shown in this study, non-frozen liposomes in 10 mM phosphate buffer without trehalose in nitrogen atmosphere degraded upon gamma-irradiation. Both the results of the chemical analysis (pH, oxidation measurements, HPLC of phospholipids) and the results of the physical analysis (DSC) suggested that trehalose reacted with species induced by gamma-irradiation and in that way reduced the irradiation damage of the liposomal bilayers. It has been reported before that sugars like hexose, fructose and glucose are not stable upon gamma-irradiation (26). No information is available about the toxicity of the degradation products formed. Even if these products are non-toxic, the use of gamma-irradiation of liposomes in the presence of trehalose is far from being elegant.

To prevent physical changes upon freeze-drying or freezing of liposomes a cryoprotectant has to be added into the dispersion (23,24,27,28). As mentioned in the Introduction, Kapanin *et al.* used 10% chlorine chloride as a cryoprotectant. However, we do not consider that this is a desirable option because then the liposome dispersion will have a extremely high osmotic value which is unacceptable for e.g. parenteral use. Until the discovery of an efficient cryoprotectant that is stable upon gamma-irradiation, reduction of the irradiation damage of liposomes by freeze-drying or freezing is an unlikely option. In pilot studies liposome dispersions without a cryoprotectant were frozen in dry-ice and exposed to gamma-irradiation. No irradiation damage was found anymore by HPLC analysis upon gamma-irradiation up to a dose of 2.5×10^4 Gy. However, under those cryoprotectant-free conditions an increase in liposome size was monitored due to aggregation or fusion of the liposomes upon freezing. This may lead to a decrease in retention in the case of water soluble compounds (29,30) and to changes in the disposition of these irradiated liposomes *in vivo*. For multilamellar, non-sized vesicles with bilayer associated drugs, fusion or aggregation of the liposomes or leakage of the drug might not pose a major problem.

In conclusion, gamma-irradiation damage of liposome dispersions can be minimised by freezing or freeze-drying in the presence of trehalose. However, trehalose acted both as a degradable scavenger and as a cryoprotectant. This degradation limits the use of freezing or freeze-drying liposome dispersions as a way to minimise irradiation damage. Future experiments should focus on the identification of a cryoprotectant that is safe and efficient under those conditions and/or the identification of a powerful antioxidant that protects the phospholipids.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support provided by Bayer A.G. (Leverkusen, FRG). The gift of the phospholipids by Nattermann Phospholipid GmbH (Cologne, FRG) and Lipoid K.G. (Ludwigshafen, FRG) was greatly appreciated. We would also like to thank Gammaster B.V. (Ede, The Netherlands) for performing of the irradiation treatments. Finally, we thank Drs. E.C.A. van Winden for the determination of the residual moisture contents of the freeze-dried liposomes.

REFERENCES

1. A.W.T. Konings. Lipid peroxidation in liposomes. In G. Gregoriadis (ed.), *Liposome Technology*, volume I, first edition, CRC Press, Inc., Boca Raton, FL, USA, 1984, pp. 139-161.

2. G. Albertini and F. Rustichelli. Effects of gamma irradiation on the liposomal structure. In G. Gregoriadis (ed.), *Liposome Technology,* volume I, second edition, CRC Press, Inc., Boca Raton, FL, USA, 1993, pp. 399-428.
3. J. Katsaras, R.H. Stinson, E.J. Kendall, and B.D. McKersie. Structural simulation of free radical damage in a model membrane system: a small-angle X-ray diffraction study. *Biochim. Biophys. Acta* **861**:243-250 (1986).
4. N.J. Zuidam, C. Versluis, E.A.A.M. Vernooy, and D.J.A. Crommelin. Gamma-irradiation of liposomes composed of saturated phospholipids. Effect of bilayer composition, size concentration and absorbed dose on chemical degradation and physical destabilisation. *Submitted for publication.*
Also published in: N.J. Zuidam. *Stability of liposomes. Chemical and physical characterisation of liposomes upon autoclaving, gamma-irradiation and storage.* Thesis, Utrecht University, Utrecht, The Netherlands, 1994.
5. R.R.C. New. Preparation of liposomes. In R.R.C. New (ed.), *Liposomes: a practical approach,* IRL Press, Oxford, UK, 1990, p. 103.
6. G. Gregoriadis. Medical uses of liposome-entrapped enzymes. *Meth. Enzym.* 44:698-709 (1976).
7. C. Brassinne, G. Atassi, J. Frühling, W. Penasse, A. Coune, J. Hildebrand, J.M. Ruysschaert, and C. Laduron. Antitumor activity of a water-insoluble compound entrapped in liposomes on L1210 Leukemia in mice. *JNCI* 70:1081-1086 (1983).
8. A. Coune, J.P. Sculier, J. Frühling, P. Stryckmans, C. Brassine, G. Ghanem, C. Laduron, G. Atassi, J.M. Ruysschaert, and J. Hildebrand. Iv administration of a water-insoluble antimitotic compound entrapped in liposomes. Preliminary report on infusion of large volumes of liposomes to man. *Cancer Treat. Rep.* 67, 1031–1033 (1983).
9. J.P. Sculier, A. Coune, C. Brassinne, C. Laduron, G. Atassi, J.M. Ruysschaert, and J. Frühling. Intravenous infusion of high doses of liposomes containing NSC 251635, a water-insoluble cytostatic agent. A pilot study with pharmacokinetic data. *J. Clin. Oncol.* 4:789-797 (1986).
10. P.V. Kapanin, N.N. Soboleva, and V.I. Trofimov. Feasibility of liposome cryoradiation sterilisation. *Khim.-Farm. Zh.* 22(4): 479-482 (1988). (in Russian language).
11. V.L. Talrose and V.I. Trofimov. The most effective way to protect drugs from ionizing radiation injury in water containing media. Publication of Institute of energy problems of chemical physics of Russ. A.S., 38/2 Leninsky prospects, Moscow, 117829, Russia.
12. F.J. Marino and F. Benjamin. Industrial sterilization. A review of current principles and practices. In K.E. Avis, L. Lachman, and H.A. Lieverman (eds.), *Pharmaceutical dosage forms: Parenteral medications,* volume 2, Marcel Dekker Inc., New York, 1984, pp. 1-54.
13. C.H. Fiske and Y. Subbarow. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375-400 (1925).
14. E.G. Bligh and W.J. Dyer. A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37(8):911-917 (1959).
15. B.J. Litman and Y. Barenholz. Fluorescent probe: diphenylhexatriene. *Meth. Enzym.* 81:678-685 (1982).
16. M. Grit, D.J.A. Crommelin, and J.K. Lang. Quantitative determination of phosphatidylcholine, phosphatidylglycerol and their lyso forms from liposome dispersions by high performance liquid chromatography (HPLC) using high sensitivity refractive index detection. *J. Chromatogr.* 585:239-246 (1991).
17. R.A. Klein. The detection of oxidation in liposome preparations. *Biochim. Biophys. Acta* 210:486-489 (1970).
18. J.H. de Smidt and D.J.A. Crommelin. Viscosity measurement in aqueous polymer solutions by dynamic light scattering. *Int. J. Pharm.* 77:261-264 (1991).
19. G. Stark. The effect of ionizing radiation on lipid membranes. *Biochim. Biophys. Acta* 1071:103-122 (1991).
20. A. Memoli, L.G. Palermiti, V. Travagli, and F. Alhaique. Lipid peroxidation of L-α-phosphatidylcholine from egg yolk and from soybean during liposome preparation and storage. *J. Liposome Res.* 3(3):697-706 (1993).
21. R.C. Bruch and W.S. Thayer. Differential effect of lipid peroxidation on membrane fluidity as determined by electron spin resonance probes. *Biochim. Biophys. Acta* 733:216-222 (1983).
22. L.I. Viera, S. Alonso-Romanowski, V. Borovyagin, M.R. Feliz, and E.A. Disalvo. Properties of gel phase lipid-trehalose bilayers upon rehydration. *Biochim. Biophys. Acta* 1145:157-167 (1993).
23. H. Talsma. *Preparation, characterization and stabilization of liposomes.* Thesis, Utrecht University, Utrecht, The Netherlands, 1991.
24. D.J.A. Crommelin, M. Grit, H. Talsma, and N.J. Zuidam. Physical stability on long-term storage. In G. Cevc (ed.), *Phospholipids Handbook,* Marcel Dekker, Inc., New York, 1993, pp. 335-348.
25. P.W. Tinsley and G. Maerker. Effect of low-dose γ-radiation on individual phospholipids in aqueous suspension. *J. Am. Oil Chem. Soc.* 70:187-191 (1993).
26. G.P. Jacobs. A review: Radiation sterilization of pharmaceuticals. *Radiat. Phys. Chem.* 26(2):133-142 (1985).
27. Y. Özer, H. Talsma, D.J.A. Crommelin, and A.A. Hincal. Influence of freezing and freeze-drying on the stability of liposomes dispersed in aqueous media. *Acta. Pharm. Technol.* 34(3):129-139 (1988).
28. G.J. Fransen, P.J.M. Salemink, and D.J.A. Crommelin. Critical parameters in freezing of liposomes. *Int. J. Pharm.* 33:27-35 (1986).
29. K. Miyajima, K. Tomita, and M. Nakagaki. Effect of saccharides on the freezing and thawing of liposome dispersions. *Chem. Pharm. Bull.* 34(7):2689-2697 (1986).
30. H. Talsma, M.J. van Steenbergen, and D.J.A. Crommelin. The cryopreservation of liposomes: 3. Almost complete retention of a water soluble marker in small liposomes in a cryoprotectant containing dispersion after a freezing/thawing cycle. *Int. J. Pharm.* 77:119-126 (1991).