Gamma-irradiation of liposomes composed of saturated phospholipids. Effect of bilayer composition, size, concentration and absorbed dose on chemical degradation and physical destabilization of liposomes

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Abstract

Liposomes composed of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), or mixtures of these two phospholipids were exposed to gamma-irradiation in an air environment. Disappearance of the mother compounds was monitored by HPLC analysis. Plotting of the logarithmic values of residual DPPC or DPPG concentration versus irradiation dose resulted in straight lines. The slopes of these lines (overall degradation constants) depended on the type of phospholipids, concentration of the liposomes and the size of the liposomes. Under the chosen conditions, addition of DPPG in DPPC-liposomes did not affect the degradation rate constant of DPPC and visa versa. The presence of phosphate buffer (pH 7.4), pH or presence of sodium chloride did not affect the irradiation damage either. Minor changes were found upon analysis of total fatty acids by GLC and upon measurement of water soluble phosphate compounds. These changes were less pronounced than the changes monitored by HPLC of phospholipids, because the HPLC analysis monitored the overall degradation of the liposomal phospholipids. Thin-layer chromatography/fast atom bombardment mass spectrometry (TLC/FAB-MS) analysis of irradiated and non-irradiated DPPC or DPPG provided information on the structure of several degradation products. Degradation routes which include these degradation products are proposed. Gamma-irradiation neither affected the size of the liposomes nor the bilayer rigidity as determined by dynamic light scattering and fluorescence anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively. However, upon gamma-irradiation, changes in the melting characteristics of the liposomes were found by differential scanning calorimetry (DSC) measurements. The pre-transition melting enthalpy of the liposomal bilayer decreased or disappeared and the main-transition broadened. The changes found in DSC scans correlated qualitatively well with the changes recorded after HPLC analysis of phospholipids.

Keywords: Gamma irradiation; Liposome; Chemical stability; Physical stability; DSC; Thin-layer chromatography/fast atom bombardment mass spectrometry; Degradation

1. Introduction

A large number of articles on the effects of irradiation on liposomes have been published (e.g., Refs. in [1,2]). The main reason for these studies is that liposomes are considered to be good model systems to gain insight into the effect of irradiation on cells and food. In spite of the large number of data that have been collected, the nature and implications of the chemical changes produced in phospholipids by gamma-irradiation are still far from clear. Interestingly, these uncertainties did not stop groups from using gamma-irradiation to sterilize liposomes [3–5], even to be used parenterally as drug carriers in man [4,5].

Organic compounds like phospholipids are affected both directly via rupture of the chemical bonds and indirectly via reactions of species induced by gamma-irradiation (in particular radicals) [2]. Not only can hydrolysis be expected, but also degradation processes such as dehydrogenation, chain rupture and dimerization. Just recently, Tinsley and Maerker demonstrated that irradiation of liposomes composed of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine or dipalmitoylphosphatidylglycerol (DPPG) resulted in the formation of dipalmitoylphosphatidic acid (DPPA) and lysophospho-
lipids [6]. However, more than half of the phospholipids that disappeared upon irradiation could not be accounted for. Also dipalmitoylphosphatidylserine and the phospholipids mentioned above containing oleic acid instead of palmitic acid, or a mixture of both fatty acids, degraded at similar amounts upon gamma-irradiation. The same authors also demonstrated the presence of traces of both isomers of palmitoylphosphocholine propanediol upon gamma-irradiation of liposomes composed of DPPC [7].

The aim of the present study was to investigate in more detail both chemical and physical changes induced by gamma-irradiation of well-defined liposome dispersions composed of saturated phospholipids in an air atmosphere and to shed light on the factors that influence these changes. This would provide basic information on the degradation pathways and amount of degradation of these phospholipids upon gamma-irradiation. To this end, liposome dispersions composed of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) or a mixture of both phospholipids were used. DPPC- or DPPG liposome dispersions containing 20 or 2 mM phospholipid were irradiated to determine the influence of phospholipid concentration. Different hydration media were used to determine the influence of phosphate buffer species (pH 7.4), pH and sodium chloride on the irradiation damage of 22 mM DPPC/DPPG 10/1-liposome dispersions.

Finally, to monitor the effect of sizing on irradiation damage and to monitor size changes after the irradiation treatment 22 mM DPPC/DPPG 10/1-liposome dispersions were also sized during their preparation process. Chemical degradation of the liposome dispersions was followed as a function of the irradiated dose by measuring the pH, the phospholipid concentration by HPLC, the total fatty acid concentration by GLC and the water soluble phospho compounds by phosphate determination. The structure of some of the degradation products was identified by thin-layer chromatography/fast atom bombardment mass spectrometry (TLC/FAB-MS) analysis. The effect of chemical degradation on the physical stability of the liposomes was assessed by measuring their size by dynamic light scattering (DLS), the thermotropic behavior by differential scanning calorimetry (DSC) and the bilayer rigidity by fluorescence anisotropy measurements of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) before and after gamma-irradiation.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were gifts from Nattermann Phospholipid (Cologne, Germany). Monopalmitoylphosphatidylcholine (LPC), lysophosphatidylglycerol (LPG) and dipalmitoylphosphatidic acid (DPPA) were obtained from Avanti Polar Lipids (Pelham, AL, USA). Palmitic acid was obtained from Sigma (St. Louis, MO, USA). These and all other chemicals were of analytical grade. The water was double-distilled before use.

2.2. 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 3 h under reduced pressure. Then the film was hydrated with 10 mM phosphate buffer (pH 7.4), or 0.13 M sodium chloride, or both 10 mM phosphate (pH 7.4) and 0.13 M NaCl. Because of unchanged pH upon gamma-irradiation (< 0.1), 10 mM phosphate buffer (pH 7.4) with or without 0.13 M NaCl was chosen as the buffer for the liposome dispersions. Other investigated buffers failed to maintain the selected pH upon gamma-irradiation. Hapes and Tris buffers (10 mM buffer and 0.13 M NaCl; initial pH 7.4) decreased 0.5 and 2.7 units in pH, respectively, after irradiation with a dose of 5.0 · 10⁴ Gy. The pH of acetate and citrate buffers (10 mM buffer and 0.13 M NaCl; initial pH 4.0) increased 0.5 and 1.1 units in pH, respectively, upon gamma-irradiation with 5.0 · 10⁴ Gy. Apart from demonstrating poor pH-stabilizing properties, the Hapes buffer also showed yellow/brown colouring after gamma-irradiation. A change in pH demonstrates that the buffer itself was affected by the gamma-irradiation. This might interfere with the experiments with liposomes.

For the preparation of dispersions denoted by ‘0.18 μm liposomes’ liposomes were extruded with a 200 ml extrusion system (Sartorius, Gottingen, Germany) through 0.6 μm and three times through 0.2 μm pore size filters, respectively (Nuclepore, Costar Corporation, Cambridge, MA, USA). For the preparation of dispersions denoted by ‘0.10 μm liposomes’ additional extrusions were performed through 0.1 μm and three times through 0.05 μm pore size filters, respectively. The maximum pressure used in the extrusion steps was 10 bar. The pH of the dispersion was measured before and after extrusion and adjusted, if necessary. The liposome dispersions were filled into 2 ml ampoules (under air).

2.3. Irradiation

The ampoules were irradiated with a ⁶⁰Co source at ambient temperature by Gammaster (Ede, The Netherlands) in small boxes at a dose rate of 7.8 · 10³ Gy/h when the samples were exposed to a dose of 1.3 · 10⁴ Gy and at a dose rate of 4.4 · 10³ Gy/h when the samples were exposed to a dose of 2.8 · 10⁴ or 5.8 · 10⁴ Gy. The dose of 5.8 · 10⁴ Gy was administered in two sessions. 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 [8])
2.4. Differential scanning calorimetry (DSC)

Liposomes were concentrated by ultracentrifugation at 200,000 x g for 30 min. The supernatant was checked for the absence of phospholipids by measuring the concentration of phosphate as described below. The pellet was put into an aluminum pan. As a reference an empty aluminum pan was used. Calorimetric scans from 30 to 60°C were performed on a Netzsch DSC 200 low-temperature DSC (Netzsch-Gerätebau, Selb, Germany). The scanning rate was 2 °C/min. The amount of phospholipids in the pan was determined by a phosphate determination in the lower phase of a Bligh and Dyer extract of the content.

2.5. Fluorescence anisotropy

To gain information about the bilayer rigidity of liposomes the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in liposomes was measured. After gamma-irradiation 5 μl of 2 x 10^{-4} M DPH, dissolved in tetrahydrofuran, was added to 25, 50 and 75 μl of liposomes (20-22 mM) diluted in buffer (total volume 3 ml). The molar ratio phospholipids/DPH was about 5, 100, 1000 and 1500, respectively. This mixture was stabilized 1 h at 60°C and protected from light. The anisotropy measurements were performed on an LS50 luminescence spectrometer (Perkin Elmer, Norwalk, CT, USA), using an excitation wavelength of 365 nm and an emission wavelength of 430 nm (slits, 5 nm). Anisotropy values (r) were computed after correction for optical and electronic differences in the parallel and perpendicular channels (G-factor). The correct anisotropy value of a dispersion was obtained after extrapolation of the anisotropy values to a liposome concentration of zero as proposed by Litman and Barenholz [9]. The temperature of the samples was 20 ± 1°C.

2.6. Analytical methods

Phospholipids were analyzed by HPLC as described earlier [10]. Samples for the HPLC analysis were prepared by the Bligh and Dyer extraction [11]. The phospholipids were collected in the chloroform phase. After dilution of the chloroform phase in methanol, 100 μl aliquots were directly injected into the column. The HPLC system consisted of a type 400 solvent delivery system (Kratos, Ramsey, N.J., USA), a Kontron sampler MSI 660 (Kontron, Zürich, Switzerland) and a Waters 410 RI detector (Waters Associates, Milford, MA, USA). Chromatograms were collected and analyzed with a computerized data system (WOW, Thermo Separation Products, Riviera Beach, FL, USA). The separation of the phospholipids was carried out on a Zorbax aminophas column (25 cm x 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 Associates, Deerfield, IL, 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/min.

The total palmitic acid content of the liposome dispersions was measured by GLC analysis basically as described by Barenholz and Amselem [12]. After a Bligh and Dyer extraction [11], an aliquot of the lower phase was transferred into an Eppendorf cup. After evaporation of the organic solvent, 50 μl tolue containing 10 mg/ml octacosan (C_{28}) and 20 μl of METH-PREP II (Alltech Associates, Deerfield, IL, USA) was added. The alkane was used as an internal standard. Palmitic acid and other acids (C_{n}-C_{26}) and alkanes (such as pentadecane and hexadecane) were used as external standards. The METH-PREP II methyl esterified the fatty acids at room temperature within 30 min. Samples were analyzed on a 1.4 m column containing 10% Silar 10C (Alltech) in a Hewlett-Packard 5710A Gas Chromatograph (Hewlett-Packard, Avondale, PA, USA). A flame ionization detector was used. Dry nitrogen was used as the carrier gas. The run was started by using an initial temperature of 100°C for 4 minutes. Then the temperature was increased at 5°C/min until it reached a final temperature of 220°C. The injection volume was 1 μl. Methyl esters were identified by comparing the retention times with those of standards.

Total concentrations of lipophilic phospho compounds were measured by phosphate determination according to Fiske and SubbaRow [13] of the lower phase of a Bligh and Dyer extraction [11] mixture. When the hydration medium consisted of 0.13 M NaCl, water soluble phospho compounds were measured by phosphate determination of the upper phase of a Bligh and Dyer extraction mixture.

Thin-layer chromatography/fast atom bombardment mass spectrometry (TLC/FAB-MS) was performed to identify possible degradation products of liposomal phospholipids upon gamma-irradiation. Chromatography was performed on precoated silica gel 60 aluminum plates (Merck, Darmstadt, Germany). 10 μl of the chloroform phase of a Bligh and Dyer extraction [11] mixture of a liposome dispersion was applied on a TLC plate. For TLC/FAB-MS analysis, the total amount of DPPC or DPPG applied to the plate was 4 x 10^{-8} or 1 x 10^{-8} mol, respectively. Plates were developed using chloroform/methanol/water/ammonia (130:35:8:4, v/v/v) as the mobile phase [14]. The running distance of the solvent from the position of sample application was 50 mm. TLC/FAB-MS was carried out by using a Jeol MS-TLCPA10 TLC/FAB probe system [15]. The system is composed of a TLC/FAB probe which supports and drives the TLC plate, a probe controller and an ion source. The TLC probe is composed of a TLC plate holder (8 mm x 55 mm) and pulse motor. The controller drives the plate in synchrony with the scan of the magnetic field. The TLC plate stops during measurement of a mass spectrum and moves 0.3 mm during the dead time between two scans. The developed plates were coated with triethanolamine as
FAB matrix before introduction into the source. Positive ion mass spectra were obtained with a Jeol JMS/SX102/102A four-sector instrument of $B_1E_1B_2E_2$ geometry. FAB mass spectra were obtained with MS-1. Xenon was used as the FAB gas; the gun was operated at 6 kV and a 5 mA discharge current. Each TLC plate was scanned twice. Tandem mass spectra were acquired by selecting the desired ion with MS-1, and colliding the selected ion at 10 keV translational energy in a collision cell at ground potential located in the third field-free region of the instrument. The ions resulting from collision-induced dissociation (CID) were recorded at a main beam reduction of 50% with air as collision gas.

The Z-average particle size and polydispersity index (p.d.) at 25°C were determined by dynamic light scattering with a Malvem 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvem, Malvern, UK). For viscosity and refractive index the values of pure water were used. The p.d. is a measure of the width of the particle size distribution and ranges from 0.0 for an entirely homogeneous, up to 1.0 for a completely heterogeneous size distribution.

2.7. Statistics

Significance tests on a mean were performed by using the Student t-test (two sided) assuming equal variances and using $\alpha = 0.05$.

3. Results

To gain insight into the factors which influence the irradiation damage a number of liposome dispersions with saturated phospholipids were made and exposed to three different doses ($1.3 \times 10^4$ Gy, $2.8 \times 10^4$ Gy and $5.8 \times 10^4$ Gy). The liposome dispersions used were: 20 and 2 mM DPPC, 20 and 2 mM DPPG and 22 mM DPPC/DPPG 10/1 (non-sized and sized to 0.18 pm (p.d. = 0.1) or to 0.10 pm (p.d. = 0.1)). The buffer consisted of 10 mM phosphate (pH 7.4) and 0.13 M NaCl. To gain insight into the influence of each component of the buffer solution on the irradiation dose, non-sized 22 mM DPPC/DPPG 10/1-liposomes were also made in either 10 mM phosphate (pH 7.4) or 0.13 M NaCl. To gain insight into the influence of each component of the buffer solution on the irradiation dose, non-sized 22 mM DPPC/DPPG 10/1-liposomes were also made in either 10 mM phosphate (pH 7.4) or 0.13 M NaCl. To gain insight into the influence of each component of the buffer solution on the irradiation dose, non-sized 22 mM DPPC/DPPG 10/1-liposomes were also made in either 10 mM phosphate (pH 7.4), or in 0.13 M NaCl (pH around neutral). The non-sized liposomes are multilamellar and the liposomes sized to 0.18 $\mu$m and 0.10 $\mu$m are oligolamellar (1 to 2 bilayers per liposome) and mainly unilamellar, respectively [16].

3.1. Chemical stability: pH, phospholipid content and degradation products

3.1.1. pH

The pH values before and after the irradiation treatment were measured and compared. The results are presented in Fig. 1. Gamma-irradiation of the liposomes resulted in a drop of the pH in all liposome dispersions. As mentioned in Section 2, phosphate buffer (pH 7.4) with or without salt did not show a change in pH upon gamma-irradiation. The dispersion with DPPG-liposomes decreased more in pH than the DPPC-liposomes upon gamma-irradiation. Upon gamma-irradiation of DPPC- and DPPC-liposomes the decrease in pH units was around 2-times larger in the 20 mM dispersions than in the 2 mM dispersions. The size of the liposomes also tended to influence the decrease in pH; the smaller the vesicles, the larger the decrease in pH. A very large pH decrease of 2 to 3 units was found in the non-buffered (0.13 M NaCl) dispersion of 22 mM DPPC/DPPG 10/1-liposomes (result not shown).

3.1.2. Phospholipid content

After gamma-irradiation, the concentrations of the phospholipids were analyzed by HPLC as described in Section 2. A typical example is shown in Fig. 2. In this figure the percentage of remaining phospholipid upon gamma-irradiation of 22 mM $0.2 \mu$m DPPC/DPPG 10/1-liposomes in 10 mM phosphate and 0.13 M NaCl is plotted against the dose semi-logarithmically. Both DPPC and DPPG decreased in concentration upon irradiation and the plots were linear. This means that degradation was not affected by the use of different irradiation procedures (different dose rates and administration of a high dose in two sessions, see Section 2). However, considerable differences were found in the slopes between different liposome batches (the same lot of lipid!), especially for the disappearance of DPPG (see Fig. 2). The same observation was also made for the other liposome dispersions (see Table 1). The rather large S.D. values in Table 1 demonstrate a considerable scattering of these data sets as well. The following observations were made upon HPLC analysis of irradiated liposomes (see Table 1). (1) The decrease in
phospholipid concentration of 20 mM DPPG liposome dispersions was larger than the decrease in phospholipid concentration of 20 mM DPPC-liposomes. The same phenomenon was observed for the 2 mM phospholipid containing dispersions (7). A small 'concentration effect' was found upon HPLC analysis of the 20 mM and 2 mM (prepared by 10 times dilution of the 20 mM dispersion with buffer) DPPG-liposome dispersions; the phospholipids of the 2 mM DPPG-liposome dispersions were relatively more degraded than the phospholipids of the 20 mM DPPG-liposomes upon gamma-irradiation. This phenomenon was not statistically significant for DPPC-liposomes.

(3) Upon gamma-irradiation DPPG in DPPC/DPPG 10/1-liposomes with a size of 0.18 μm (p.d. = 0.1) and 0.10 μm (p.d. = 0.1) was more degraded than DPPG in the non-sized vesicles. For DPPC no 'size effect' was observed. (4) Upon gamma-irradiation the same degree of degradation of DPPC and DPPG was observed in liposomes composed of only one of the phospholipids (20 mM DPPC-liposomes and 2 mM DPPG-liposomes, respectively) and in the liposome dispersion composed of the mixture of both phospholipids (22 mM DPPC/DPPG 10/1-liposomes). Apparently, the degradation of one type of phospholipid was not affected by the presence of the other type. (5) In Table 1 it is also shown that leaving out the 10 mM phosphate buffer or 0.13 M NaCl did not affect the irradiation damage of DPPC or DPPG. Apparently, under the chosen conditions pH (in the region 4 to 7.4 as found with non-buffered 22 mM DPPC/DPPG 10/1-liposomes, see above) and ionic strength had no influence on the damaging effect of gamma-irradiation on liposomes. In Table 1 we have included the theoretical percentage degradation for the liposome dispersion when exposed to a sterilizing dose of gamma-irradiation (2.5 × 10⁴ Gy) as is necessary to comply with pharmacopeial standards.

3.1.3. Degradation products

Hardly any signs for degradation products of the irradiated liposomal phospholipids were detectable in the HPLC analysis used. In the HPLC system used, DPPC as well as DPPG and its lysophospholipids can be analyzed [10]. Interestingly, HPLC analysis of a mixture of different species of phosphatidy cholines also resulted in broadening or splitting of the PC peak. Splitting of the PC peak could already be observed with a mixture (1:1) of DPPC and dilauryl (12:0) phosphatidylcholine. Upon HPLC analysis of phospholipids of irradiated liposomes neither splitting nor broadening of the DPPC or DPPG peak, nor appearance of peaks other than lysophosphatidylcholine (LPC) was found. Sometimes LPC in liposome dispersions composed of 20 mM DPPC or 22 mM DPPC/DPPG 10/1 was observed after exposure to higher irradiation doses. Then, the concentrations of LPC were always close to the detection limit (= 2 nmol) of the HPLC system used. This latter finding indicated that the concentration of LPC was not higher than about 1 mol% of the DPPC content. Thus, degradation of DPPC and DPPG does not seem to result in formation of a major fraction of phospholipids with shorter lives. 

Table 1

<table>
<thead>
<tr>
<th>Liposome dispersion</th>
<th>Concentration (mM)</th>
<th>Size (p.d.)</th>
<th>Hydration medium</th>
<th>Slope for DPPC (10⁻⁶ Gy⁻¹)</th>
<th>Slope for DPPG (10⁻⁶ Gy⁻¹)</th>
<th>Theoretical degradation (% of DPPC at 2.5 × 10⁴ Gy)</th>
<th>Theoretical degradation (% of DPPG at 2.5 × 10⁴ Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC 20</td>
<td>non-sized</td>
<td>P + NaCl</td>
<td>−2.4 ± 0.6</td>
<td>−</td>
<td>6 ± 1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DPPC 2</td>
<td>non-sized</td>
<td>P + NaCl</td>
<td>−3.0 ± 0.3</td>
<td>−</td>
<td>7 ± 1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DPPG 20</td>
<td>non-sized</td>
<td>P + NaCl</td>
<td>−4.8 ± 0.8</td>
<td>−</td>
<td>11 ± 2</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DPPG 2</td>
<td>non-sized</td>
<td>P + NaCl</td>
<td>−8.1 ± 0.3</td>
<td>−</td>
<td>18 ± 1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DPPC/DPPG 10/1</td>
<td>22</td>
<td>non-sized</td>
<td>−2.4 ± 0.2</td>
<td>−8.3 ± 1.2</td>
<td>6 ± 1</td>
<td>−19 ± 3</td>
<td>−</td>
</tr>
<tr>
<td>DPPC/DPPG 10/1</td>
<td>22</td>
<td>0.10 μm (0.1)</td>
<td>−3.8 ± 1.5</td>
<td>−22 ± 9</td>
<td>9 ± 4</td>
<td>42 ± 20</td>
<td>49 ± 20</td>
</tr>
<tr>
<td>DPPC/DPPG 10/1</td>
<td>22</td>
<td>non-sized</td>
<td>−4.3 ± 2.2</td>
<td>−20 ± 8</td>
<td>10 ± 5</td>
<td>52 ± 18</td>
<td>59 ± 18</td>
</tr>
<tr>
<td>DPPC/DPPG 10/1</td>
<td>22</td>
<td>non-sized</td>
<td>−2.0 ± 0.5</td>
<td>−10 ± 5</td>
<td>5 ± 1</td>
<td>22 ± 11</td>
<td>−</td>
</tr>
<tr>
<td>DPPC/DPPG 10/1</td>
<td>22</td>
<td>non-sized</td>
<td>−2.8 ± 0.9</td>
<td>−10 ± 1</td>
<td>7 ± 2</td>
<td>23 ± 2</td>
<td>−</td>
</tr>
</tbody>
</table>

Data represent the mean of triplicate-experiments ± S.D.

* The hydration media of the dispersions consisted of 10 mM phosphate (pH 7.4) (P) and/or 0.13 M NaCl.
fatty acids chains upon gamma-irradiation and the concentrations of LPC in all dispersions were always low (≤ 1 mol%).

Total fatty acid analysis with GLC demonstrated that irradiation of liposomal DPPC and DPPG did not result in a large decrease in total concentration of palmitic acids. Other compounds than palmitic acids were not found in the samples of irradiated liposomes, e.g., shorter fatty acid chains (down to about capric acid (C_{10})), pentadecane or hexadecane. For example, upon gamma-irradiation of 22 mM DPPC/DPPG 10/1-liposomes (those were the dispersions that were most sensitive to gamma-irradiation among the dispersions studied, see Table 1), the percentage of remaining total palmitic acid decreased only significantly after exposure to the highest irradiation dose of 5.8 · 10^4 Gy: 93 ± 4% (relative to starting condition; other results not shown). Under those circumstances the percentages 'remaining DPPC and DPPG' were 79 ± 6% and 15 ± 14%, respectively. Although the percentage of degradation of palmitic acid was only 7% of the initial concentration of 44 mM, these data indicate that at most 50% of phospholipids that disappeared (27% of total) can be accounted for by monitoring palmitic acid degradation (100% - 2 · 7%/27% ≈ 50%). This maximum of 50% is based on the assumption that degradation of only one palmitic acid unit is the only degradation process that a particular phospholipid is undergoing.

In all dispersions the concentration of total lipophilic phospho compounds was measured by phosphate determination in the lower phase of a Bligh and Dyer extraction mixture of the samples. No significant decrease could be found upon irradiation. Upon gamma-irradiation of 22 mM DPPC/DPPG 10/1-liposomes in only 0.13 M NaCl a slight, but statistically significant increase in the percentage of water soluble phospho compounds was only found after exposure of the dispersions to a dose of 2.8 · 10^3 and 5.8 · 10^4 Gy; 0.9 ± 0.2 and 1.9 ± 0.7% of the total phosphate present, respectively, could be recovered in the upper phase of a Bligh and Dyer extract.

Thin-layer chromatography/fast atom bombardment mass spectrometry (TLC/FAB-MS) was performed to identify possible degradation products of liposomal phospholipids upon gamma-irradiation. Aliquots of the chloroform phase of a Bligh and Dyer extract of 20 mM DPPC- or DPPG-liposomes were applied on precoated silica gel 60 aluminum plates and developed as described in Section 2. One of the plates was sprayed with a solution of molybdenum blue to obtain dark-blue spots of phosphate containing compounds (see Fig. 3). Similar, non-sprayed TLC plates were used for TLC/FAB-MS measurements (see Fig. 4A,B and Table 2). In Fig. 4, a typical example of the TLC/FAB-MS analysis is shown. In mass chromatograms of non-irradiated DPPG with m/z value of 745 and 671, only one peak around scan number 75 can be observed (see Fig. 4A). Apparently, FAB ionization of DPPG resulted in molecular ions ([M + Na]^+; m/z 745) and fragment ions, most likely [M + Na - C_{3}H_{6}O_{2}]^+ at m/z 671. The peak around scan number 75 also appeared in both selected mass chromatograms of the irradiated DPPG (Fig. 4B). However, in the mass chromatogram with m/z value of 671, an additional peak at around scan number 25 can also be observed (see Fig. 4B). This additional peak was not present in the mass chromatogram of non-irradiated DPPG with m/z value of 671 (see Fig. 4A). Thus, this is evidence for a degradation product as result of gamma-irradiation of DPPG, i.e., DPPA ([M + Na]^+). Apparently, molecular ions of the degradation products as resulting from gamma-irradiation can be identical to fragment ions resulting from FAB ionization, because of the use of high energy particles. However, these ions can be distinguished from each other, because they will be detected at different positions (= scan numbers in the mass chromatogram) at the TLC-plate after gamma-irradiations. The other results are shown in Table 2.

Fig. 3. Picture of a developed TLC-plate sprayed with molybdenum blue (blue spots). The following samples were applied: (1) standard LPC, (2) standard LPG, (3) standard DPPA, (4) standard DPPC, (5) standard DPPG, (6) non-irradiated DPPC, (7) irradiated DPPC (5.8 · 10^4 Gy), (8) non-irradiated DPPG and (9) irradiated DPPG (5.8 · 10^4 Gy). The amounts of the standards and samples applied to the plate were 2 · 10^{-3} mol and 1.6 · 10^{-1} mol, respectively. The spots represent probably the presence of (a) LPC, (b) LPG, (c) DPPA, (d) DPPC, (e) DPPG, (f) 1- or 2-palmitoyl-sn-propanediol-3-phosphorylcholine, (g) 1- or 2-palmitoyl-sn-propanediol-3-phosphorylglycerol, (h) dipalmitoyl-sn-glycerol-3-phosphoryl-(1,2-dihydroxyacetone) or dipalmitoyl-sn-glycerol-3-phosphoryl-(1,2-dihydroxypropaldehyde) and (i) dipalmitoyl-sn-glycerol-3-phosphorylethanol. Other spots are unknown. See text, Table 2 and Fig. 6 for further information.
### Table 2
TLC/FAB-MS analysis of 20 mM non-sized DPPC- or DPPG-liposomes before or after gamma-irradiation

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Dose (Gy)</th>
<th>m/z value</th>
<th>Compounds present before gamma-irradiation</th>
<th>Degradation products upon gamma-irradiation</th>
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a See also text and Fig. 4 for explanation of the TLC/FAB-MS analysis.

b No attempts were made to interpret the data quantitatively, because the intensity of the observed peaks depends on many factors such as matrix, surface activity of the ions, ionization of the compounds, etc.

c Additional measurements of sodium adduct ions, proton adduct ions, matrix adduct ions, sodium matrix adduct and/or proton matrix adduct ions at the same scan position confirmed the presence of all the proposed ions. See also Fig. 6 for the structure of the proposed molecular ions.

d As demonstrated by HPLC analysis, LPC is probably another degradation product although the TLC/FAB-MS data do not show explicitly an increase in LPC level upon gamma irradiation.
Fig. 4. Selected mass chromatograms of non-irradiated and irradiated DPPG as a typical example of the TLC/FAB-MS analysis of non-irradiated and irradiated DPPC or DPPG. The m/z values of the selected mass chromatograms are 745 and 671. The values at the x-axis indicate the relative length of a lane of the TLC-plate (such as shown in Fig. 3). The values at the y-axis indicate the relative value of the ion current. (A) Non-irradiated DPPG; (B) irradiated DPPG (5.8 \times 10^4 Gy). See also text.

2. See also Fig. 6 (Section 4) for the structure of the proposed molecular ions in Table 2.

Upon gamma-irradiation of DPPC to a dose of 5.8 \times 10^4 Gy the following degradation products were found: DPPA and 1 or 2-palmitoyl-sn-propanediol-3-phosphorylcholine. The detection of these two compounds at around the same scan number (see Table 2) is not a problem for the identity propositions, because it is difficult to envision how one can result from the other (see also Section 4 and Fig. 6 below for proposed degradation routes). LPC was detected in both the non-irradiated and irradiated sample. Apparently, a small amount of LPC is present even when no gamma-irradiation is applied (probably resulting from hydrolysis during preparation, storage and analysis). LPC is probably another degradation product, but our TLC/FAB-MS data do not show explicitly an increase in LPC level upon gamma-irradiation. However, HPLC analysis demonstrated the generation of a small fraction of LPC after gamma-irradiation (see above). TLC/FAB-MS analysis of irradiated DPPG (dose = 5.8 \times 10^4 Gy) probably showed the presence of the following degradation products: DPPA, dipalmitoyl-sn-glycerol-3-phosphoryl-(1,3-dihydroxyacetone) or dipalmitoyl-sn-glycerol-3-phosphoryl-(1,2-dihydroxypropaldehyde), LPG, 1 or 2-palmitoyl-sn-propanediol-3-phosphorylglycerol and dipalmitoyl-sn-glycerol-3-phosphorylethanol. The chemical structure of the last compound was confirmed by TLC/FAB-MS-MS: the mass spectrum of dipalmitoyl-sn-glycerol-3-phosphorylethanol was similar to a mass spectrum of DPPG, if the mass difference in headgroup was taken into account. The detectable amount of the degradation product monitored in the mass chromatogram of m/z 743 around scan number 38 was not sufficient to perform a proper TLC/FAB-MS-MS analysis.

The structure of the degradation products proposed above were in agreement with other selected mass chromatograms of possible fragment ions (peaks around the same scan numbers). The elution positions on the TLC plate were also in line with the proposed structures: more polar compounds had a lower Rf value. The proposed structural identity of DPPC, LPC, DPPG, DPPA and LPG was also obtained by comparing their elution position on the TLC plate compared to those of standards (see Fig. 3). Other, theoretically possible degradation products (e.g., palmitic acid, see also Section 4 and Section 5) upon gamma-irradiation of liposomal DPPC or DPPG could not be identified with the TLC/FAB-MS procedure used.

3.2. Physical stability: size, bilayer rigidity and thermotropic behavior

The effect of chemical degradation on the physical stability of the liposomes was assessed by monitoring the size, the thermotropic behavior and the bilayer rigidity before and after gamma-irradiation.

3.2.1. Size

Dynamic light scattering provides data that can be properly evaluated only if narrow particle size distributions (e.g., extruded lipid dispersions) are dealt with. Extrusion of 22 mM DPPC/DPPG 10/1-liposomes as described in Section 2 resulted in liposomes with an average size of 0.18 \mu m (p.d. = 0.1) and in liposomes with an average size of 0.10 \mu m (p.d. = 0.1). Hardly any change in size could be found upon gamma-irradiation. Only after exposure to a dose of 5.8 \times 10^4 Gy did one of the three 0.1 \mu m DPPC/DPPG 10/1-liposome dispersions change in size characteristics (not irradiated: 0.10 \mu m, p.d. = 0.12; 5.8 \times 10^4 Gy: 0.16 \mu m, p.d. = 0.46). This could be attributed to the complete disappearance of DPPG (determined by HPLC) during the irradiation process in just this particular dispersion.

3.2.2. Bilayer rigidity

Fluorescence anisotropy measurements of DPH in bilayers were used to monitor the fluidity of these bilayer upon gamma-irradiation. The anisotropy values of DPH of 20 mM DPPC, 20 mM DPPG, 22 mM DPPC/DPPG 10/1-liposomes were 0.321 \pm 0.002, 0.359 \pm 0.005 and 0.354 \pm 0.010, respectively. Sizing of the 22 mM DPPC/DPPG 10/1-liposomes to 0.10 or 0.18 \mu m (p.d. = 0.1) did not affect the anisotropy values. The concentrations of the liposomes given here are the concentrations during gamma-irradiation. In all dispersions the changes in anisotropy values of DPH upon gamma-irradiation were small and no consistent trend could be observed.

3.2.3. Thermotropic behavior

After gamma-irradiation the thermotropic behavior of the liposomes changed as measured by DSC. As typical
profiles of DPPC and DPPG as a function of the dose in the semilogarithmic plots. The HPLC analysis used in the present study demonstrated differences between several liposome batches used (see Fig. 2). Thus, intra-batch variation is small. However, inter-batch comparison showed unusual large variations. Such large differences between liposome batches are uncommon in hydrolysis studies for liposome dispersions [17,18] and can not be explained by small differences in phospholipid concentrations between the different batches (around 5%). This observation indicates that we had not identified, and did not control, all critical factors influencing irradiation damage. This variation can not be attributed to different degrees of contamination of the buffer by, for example, metals; the same buffer was used to prepare the three liposome preparations. Moreover, it was found that addition of EDTA (unpublished results) or Fe ions (Ayelet Samuni, Hebrew University, Jerusalem; results not published yet) did not change the irradiation damage. It has been found before that metal ions increase the irradiation damage (reviewed in [1]). However, in those studies oxidation was followed at very low irradiation doses (about 10–100 Gy) compared with the doses used in the present study.

HPLC analysis of liposomal phospholipids demonstrated that under the chosen conditions the irradiation damage depends (1) on the type of phospholipid, (2) concentration of the liposomes and (3) the size of the liposomes and not (4) on the presence of the other type of phospholiposomal (DPPC or DPPG, mutatis mutandi) nor on the (5) pH, presence of phosphate buffer (pH 7.4) and presence of sodium chloride concentration (see Table 1).

(1) Upon gamma-irradiation liposomal DPPG degraded more than DPPC (see Fig. 2 and Table 1). Tinsley and Maerker also reported that non-sized DPPG-liposomes were less stable than non-sized DPPC-liposomes (25.8% and 2.4% degradation, respectively; concentration of liposomes 5 mM) after exposure to a dose of 9.66 kGy [6].

(2) We demonstrated that the relative loss of DPPG in 2 mM DPPG-liposome dispersions was relatively larger than for 20 mM DPPG-liposomes after gamma-irradiation (see Table 1). This concentration dependency was not so clear cut with DPPC-liposomes, but a similar tendency could be observed. On the basis of $^1$H-NMR analysis of irradiated egg yolk lecithin liposomes, Sprinze et al. also reported a relatively larger irradiation damage upon dilution of the liposome dispersion [19].

(3) In this study, a size effect was observed upon irradiation of 22 mM DPPC/DPPG 10/1-liposomes of different sizes; more DPPG disappeared with the 0.10 or 0.18 μm liposomes than with the non-sized vesicles which are probably at least 10 times larger (see Table 1). A difference in oxidation damage between liposomes of difference size was also reported by Petkau and Chelack [20]. Sonicated liposomes (average size 21 ± 5 nm) composed of soybean lipids or composed of phospholipids from fresh beef brain were more oxidized than non-sonicated vesicles after exposure to low irradiation doses.

4. Discussion

4.1. Chemical stability: phospholipid content, pH and degradation products

4.1.1. Phospholipid content

As shown in Fig. 2 and Table 1 HPLC analysis of phospholipids used in this study is a powerful analytical tool to monitor the irradiation damage to liposomes. As shown for the first time in the present study, per liposome batch (of the same lot of phospholipid) the disappearance profiles of DPPC and DPPG as a function of the dose absorbed were consistent, as indicated by the linearity of the semilogarithmic plots. The HPLC analysis used in the
Table 3
The melting characteristics of non-irradiated and irradiated liposomes

<table>
<thead>
<tr>
<th>Liposome dispersion</th>
<th>Size (p.d.)</th>
<th>Dose ((10^1 \text{ Gy}))</th>
<th>(\Delta H_m) ((\text{kJ/mol P}))</th>
<th>(T_{on}) (^{(\circ} \text{C}))</th>
<th>(T_{on}) (^{(\circ} \text{C}))</th>
<th>(\Delta T_{1/2}) ((\circ))</th>
<th>(\Delta H_p) ((\text{kJ/mol P}))</th>
<th>(T_m) (^{(\circ} \text{C}))</th>
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</thead>
<tbody>
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<td>20 mM DPPC</td>
<td>non-sized</td>
<td>0</td>
<td>31 ± 1</td>
<td>41.6 ± 0.1</td>
<td>42.2 ± 0.4</td>
<td>0.9 ± 0.1</td>
<td>3.5 ± 0.6</td>
<td>37.0 ± 0.2</td>
</tr>
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<td>non-sized</td>
<td>1.3</td>
<td>32 ± 2</td>
<td>41.6 ± 0.1</td>
<td>42.3 ± 0.3</td>
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<td>2.7 ± 0.2</td>
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<td>30 ± 2</td>
<td>42.0 ± 0.2</td>
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<tr>
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<td>30 ± 3</td>
<td>41.7 ± 0.2</td>
<td>47.4 ± 0.2</td>
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<td>36 ± 0.2</td>
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<td>22 mM DPPC/DPPG 10/1</td>
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<td>1.3</td>
<td>33 ± 1</td>
<td>41.8 ± 0.4</td>
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<td>5.8</td>
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<td>40.5 ± 0.4</td>
<td>43.4 ± 0.3</td>
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<td>22 mM DPPC/DPPG 10/1</td>
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<td>41.6 ± 0.1</td>
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<td>22 mM DPPC/DPPG 10/1</td>
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<td>34 ± 1</td>
<td>40.4 ± 0.9</td>
<td>43.8 ± 0.4</td>
<td>5 ± 3</td>
<td>nd</td>
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</tbody>
</table>

The buffer of all dispersions consisted of 10 mM phosphate and 0.13 M NaCl. Data represent the mean of triplicate experiments ± S.D.

The concentrations of the liposomes given here are the concentrations during gamma-irradiation.

- \(\Delta H_m\) is the enthalpy of the \(P_{60} \rightarrow L_m\) transition, the so-called main phase transition.
- \(\Delta H_p\) is the enthalpy of the \(L_{m} \rightarrow P_{60}\) 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 peak.
- \(\Delta T_{1/2}\) is the width of the main phase transition at half height.
- P, phosphate.
- nd, not detectable by the method used.
(4) With DPPC and DPPG, the process of irradiation damage to one of these bilayer constituting phospholipids did not seem to be affected by the presence of the other lipid.

(5) The presence of phosphate buffer, the presence of sodium chloride and pH (not constant in the non-buffered liposome dispersions) did not influence the irradiation damage under the conditions studied (see Table 1).

The latter two findings (absence of bilayer charge dependency and no influence of ionic strength on the irradiation damage under the conditions studied) suggest that electrostatic interactions between phospholipids and charged species induced by gamma-irradiation (e.g., hydrated electrons and protons [1]) do not play a major role in the degradation process. This agrees with results published by Barber and Thomas [21]. They described that the irradiation damage of liposomes composed of distearoylphosphatidylcholine is due mainly to the indirect action of \( \text{OH}^\cdot \) radicals with the bilayers.

4.1.2. pH and degradation products

To gain insight into the effect of irradiation on cells and food and to properly judge the possibility to use gamma-irradiation as a sterilization method for liposomes, it is important to know the major degradation routes and major degradation products upon such treatment. The reaction(s) of gamma-irradiation induced species with liposomes resulted in the formation of protons (see Fig. 1). However, only a qualitative relation between drop in pH and the results of the HPLC analysis of phospholipids could be found. Interestingly, other experiments showed that upon gamma-irradiation of liposomes in a nitrogen atmosphere the pH did not change, while the drop in phospholipid concentration as determined by HPLC analysis was similar to the result reported here [22].

Hardly any evidence for the presence of lysophosphatidylcholine (\(< 1\%\)) and water soluble glycerophospho compounds was found (\(< 2\%\)), indicating that hydrolysis reactions as observed on storage [17,18,23] are not a major degradation route when gamma-irradiation is applied. The presence of a low lysophosphatidylcholine content after gamma-irradiation of DPPC-liposomes agrees with earlier findings [6,24].

GLC analysis of the total palmitic acid content of a liposome dispersion showed a small decrease in palmitic acid upon gamma-irradiation (see Section 3). From our data, no direct correlation could be established between palmitic acid loss and disappearance of phospholipids. Upon exposure of 5 mM DPPC-liposomes to a dose of 9.66 kGy, Tinsley and Maerker [6,7] reported the presence of 0.6 mol% LPC, 0.4 mol% DPPA and traces (amount not mentioned) of both isomers of palmitoylphosphatidylcholine propanediol (in the present study called 1 or 2-palmitoyl-sn-propanediol-3-phosphorylcholine). DPPA was also present after irradiation of 5 mM DPPG (19.6 mol%). In the present study, additional evidence was collected for the presence of these degradation products upon gamma-irradiation of 20 mM DPPC-liposomes by using TLC/FAB-MS analysis (see Table 2). As shown (to our knowledge) for the first time in the present study, similar degradation products were formed upon gamma-irradiation of 20 mM DPPG-liposomes. We also observed the presence of other degradation products which have, to our knowledge, never been reported before after gamma-irradiation of 20 mM DPPG-liposomes (see Table 2). The possible structures are 1,2-dipalmitoyl-sn-glycerol-3-phospho-(1,3-dihydroxyacetone), dipalmitoyl-sn-glycerol-3-phosphoryl(1,2-dihydroxypropanediol), LPG, 1 or 2-palmitoyl-sn-propanediol-3-phosphorylglycerol and 1,2-dipalmitoyl-sn-glycerol-3-phosphorylethanol. This is the first time that degradation of the headgroup of a phospholipid has been demonstrated upon gamma-irradiation. We would like to stress that these products are only identified by one method. No effort has yet been made to quantify the trace amounts of these compounds.

The presence of these products can be explained by studying proposed degradation pathways published after gamma-irradiation experiments of dry tripalmitin, dipalmitoylphosphatidylethanolamine (DPPE) and sugars (sugars contain hydroxyl groups like DPPG) [25-27]. Two of these studies also showed that gamma-irradiation of dry DPPE may lead to a large number of different volatile and non-volatile degradation products such as alkanes, alkenes, alkanones, ethanolamine phosphate, etc. [25,27]. The possible degradation routes which may lead to the different degradation products found in the present study upon gamma-irradiation of liposomal DPPG found are shown in Fig. 6. Except for the degradations which take place in the headgroup, DPPC will probably follow the same degradation routes upon gamma-irradiation (as shown in Fig. 6A).

4.2. Physical stability: size, thermotropic behavior and bilayer rigidity

4.2.1. Size and bilayer rigidity

The size of the liposomes was not affected by gamma-irradiation, unless all DPPG was degraded. The changes in anisotropy values of DPH in all dispersions were small. In contrast with our data, Ianzini et al. reported an increase in anisotropy upon gamma-irradiation of multilamellar DPPC and DSPC-liposomes [28]. They suggested that (non-proven) crosslinking of lecithin radicals formed by the OH radical attack on the fatty acids may be responsible for this phenomenon. Part of the discrepancy between their and our results may be ascribed to differences in measuring techniques. In the present study the anisotropy value is reported after extrapolation of the measured anisotropy values to a liposome concentration of zero as proposed by Litman and Barenholz ([9] and Barenholz, personal communication). Increase in liposome concentration can result in a decrease of the anisotropy value due to scattering of the exciting light by the liposomes in the cuvette [29,30].
Fig. 6. Proposed degradation routes for liposomal DPPG in the phosphatidyl moiety (A) and in the headgroup (B) upon gamma-irradiation.

Ianzini et al. did not describe such a correction. Correction is necessary, especially because the amount of scattering decreased gradually upon gamma-irradiation (11 ± 1% decrease in turbidity of 20 mM DPPC-liposomes at 430 nm after 5.8 · 10^4 Gy), probably due to changes in chemical composition of the bilayers. Upon gamma-irradiation, Ianzini et al. also reported a large drop in the fluorescence yield of DPH (added after gamma-irradiation) [28]. In the present study a much smaller decrease (≤ 10%) was observed. This drop could possibly be due to chemical changes in the bilayers. However, here again, a difference in scattering behavior of the liposome dispersions might contribute to this discrepancy as well. Another possibility is that degradation of the 15% sucrose solution used in their liposome dispersion interfered with their experiments.

4.2.2. Thermotropic behavior

Of the physical techniques used in this study only the DSC experiments showed pronounced changes in physical characteristics of liposomes upon gamma-irradiation (see Fig. 5 and Table 3). The melting characteristics of the non-irradiated liposomes are in good agreement with data reported in the literature [31]. Sizing resulted in small changes in the shape of the peak of the main phase transition and a decrease in the ΔH_p as was found before [32]. Upon gamma-irradiation the pre-transition decreased or disappeared. The pre-transition has been reported to be highly sensitive to perturbations of the lipid matrix [33]. The main phase transition broadened and showed a shoulder at the right-hand side, indicating the presence of components with other thermotropic behavior. Of the non-sized liposomes the 20 mM DPPC-liposomes were less affected than the other non-sized liposomes. The sized liposomes showed a more pronounced broadening of the main-transition than the non-sized liposomes upon gamma-irradiation. As shown for the first time in this study, the observed physical changes as determined by DSC correlated qualitatively well with the chemical degradation as monitored by HPLC analysis of phospholipids (compare Tables 1 and 3). The DSC scans did not change upon repeated scanning of the same sample (two cycles), indicating that the observed changes in thermotropic behavior had a permanent character. The observed changes in the DSC scans of liposomes composed of saturated phospholipids after gamma-irradiation are similar to the results reported before [2,24,28,34]. The broadening of the peaks of the main phase transition might be (partly) explained by the presence of the lipophilic degradation products mentioned above, which will accumulate in the bilayer. For example, liposomes composed of only DPPA have a melting temperature of 68°C [30].

4.2.3. Mechanism(s) of irradiation damage of liposomes

Sprinz et al. described on the basis of ^1H-NMR measurements a preferred irradiation damage of the outer bilayer of small sonicated egg yolk lecithin vesicles (5
wt%, ~ 67 mM) after exposure to a dose of 13.5 kGy in air atmosphere [18]. They suggested that a preferred irradiation damage of the outer layer might be caused by the specific geometric packing properties of small vesicles. It is well known that in very small vesicles the surface area per phospholipid exposed is larger for the phospholipid in the outer layer than for the phospholipids in the inner layer [35]. In the present study, we used larger liposomes (about 0.10 and 0.18 μm) with no pronounced differences in area per phospholipid at the inner or outer leaflet. Therefore, our data suggest that other mechanism(s) should be involved. Another hypothesis described by Sprinz et al. is asymmetric damaging of the bilayers due to the difference in the volume of water outside and inside the liposomes. The generation of products by water radiolysis (e.g., radicals) causes most of the damaging effect for liposome bilayers [1]. Assuming that the number of generated damaging species (mainly OH') radicals) is constant per unit volume, a much higher number of species will be produced in the outer bulk water than inside the vesicles. At the lipid concentration used by Sprinz et al., each OH' radical from the bulk water will have a lifetime long enough to be able to interact with the surface of the liposome and only a small fraction of radicals can penetrate through the outside layer (because the OH' radical reacts efficiently with bilayers after collision [21]). This hypothesis might be an explanation for the observed size and concentration effect of multimamellar liposomes in the present study (see Table 1). In a non-sized, multilamellar liposome dispersion a relatively low fraction of phospholipids is exposed to the outside, 'bulk' water phase compared to dispersions containing sized liposomes [36]. Liposomes with an average size of 0.10 μm are mainly unilamellar and 0.18 μm liposomes are oligolamellar (1 to 2 bilayers per liposome). In these sized liposomes a higher fraction of phospholipids (up to 50%) is exposed to the outside. However, some of our data appear to be in conflict with this concept, as they can not explain the similar degree of DPPG degradation in 22 mM DPPC/DPPG 10/1-liposomes and in 2 mM DPPG-liposomes (see Table 1). Our data suggest that irradiation damage is not so much correlated with the total amount of radicals, but more with the fraction of radicals that is generated in the environment of the liposomal bilayers. In contrast with Sprinz et al., we hypothesized that not all OH' radicals are able to diffuse from the bulk to the bilayers, since their lifetimes are too short. It is clear that more data, e.g., on the lifetime and operational range of radicals, are required to fully understand the parameters that control the complex process of degradation of liposomal phospholipids by gamma-irradiation.

5. Conclusions

Liposomes composed of saturated phospholipids in an air atmosphere were affected by gamma-irradiation as could be shown by chemical and physical analysis. HPLC analysis of irradiated liposomal phospholipids showed that the irradiation damage depended on the type of phospholipid (DPPC < DPPG), concentration of the liposomes (20 < 2 mM) and the size of the liposomes (non-sized < 0.18 μm < 0.10 μm) and not on a competitive interaction between liposomal phospholipids (DPPC and DPPG), nor on the presence of phosphate buffer (pH 7.4), pH and presence of sodium chloride. DSC measurements monitored dose dependent changes in the melting characteristics of the liposomes upon gamma-irradiation. Other chemical techniques (GLC of fatty acids and determination of the concentrations of total water soluble and total lipophilic phosphate compounds) or physical techniques (size measurements and fluorescence anisotropy of DPH) hardly demonstrated any changes after gamma-irradiation of the liposomes. TLC/FAB-MS(-MS) analysis shed light upon some of the degradation products present after gamma-irradiation of 20 mM DPPC- or DPPG-liposomes.

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