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Physical (in) stability of liposomes upon chemical hydrolysis: the role of lysophospholipids and fatty acids

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Abstract

As a consequence of chemical hydrolysis of liposomal phospholipids the organization of the lipid assembly can change from a lamellar into a micellar system. Different approaches provided evidence for this conversion: ^{31}P-NMR analysis, turbidity measurements and ultracentrifugation experiments. Two conditions have to be met before this conversion can take place: (1) the liposomes must pass through a gel-to-liquid crystalline phase-transition during a heating or cooling run, and (2) the degree of chemical hydrolysis must exceed a critical hydrolysis percentage (or the phospholipid bilayer must contain critical amounts of lysophospholipid and fatty acid). As monitored by turbidity measurements, this critical level of hydrolysis and the relative change depended on the chain length and on the head group of the liposomal phospholipids. It does not depend on concentration, pH, storage temperature or on size of the liposomes within the experimental range. Addition of cholesterol to bilayers composed of dipalmitoylphosphatidylcholine prevents the lamellar to micellar transformation. Fluorescence anisotropy measurements of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene in 0.18-μm dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol (10:1)-liposomes indicated that behavior of the probe below and above the phase-transition temperature was not affected by chemical hydrolysis, or even by formation of micelles. However, the phase-transition temperature range broadened and shifted towards higher temperatures upon hydrolysis.

Keywords: Liposome; Chemical and physical stability; Lysophospholipid; Fatty acid; Micelle; Chemical hydrolysis

1. Introduction

Liposomes are used as carriers for drugs and diagnostic agents [1,2]. In general, in early stages of development, freshly prepared liposomes are used. However, from a pharmaceutical point of view it is important to demonstrate that liposomes can be stored for a long period of time. Degradation processes, in particular oxidation and hydrolysis, may change the properties of an aqueous liposome dispersion [3]. Oxidation of phospholipids is probably not a major problem, since it can be minimized by preventive and protective measures such as the use of antioxidants or an inert atmosphere [4]. More problematic for the long-term chemical stability of aqueous liposome dispersions is the chemical hydrolysis of ester glycerophospholipids to free fatty acids, lysophospholipids and glycerophospho compounds [3,5–8]. Formation of substantial amounts of these hydrolysis products may lead to an increase in particle size [9], to an increase in permeability of liposome bilayers [10] and also to various toxic effects [11]. To our knowledge, no other data about the effect of 'aging' on the liposome structure and stability are available in the literature. This is the subject of our current study.

Abbreviations: CHOL, cholesterol; DLS, dynamic light scattering; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; FA, palmitic acid; GPC, glycerophospho compounds; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; r, fluorescence anisotropy value; T_m, main phase-transition temperature; TPA, *trans*-parinaric acid.

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Liposome dispersions differing in composition, charge, size and pH were prepared and stored at 30 or 70°C. No drugs or model compounds were added in order to have general applicability. Physical stability of liposomes as a function of chemical hydrolysis was monitored with different techniques: measurement of particle size by dynamic light scattering (DLS), measurement of turbidity at 450 nm, assessment of bilayer rigidity by fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and organization of the lipid assembly by ^{31}P-NMR measurements and ultracentrifugation experiments.

2. Materials and methods

2.1. Materials

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) were gifts from Nattermann Phospholipid (Cologne, Germany). Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were gifts from Lipoid (Ludwigshafen, Germany). Cholesterol (CHOL) and palmitic acid (FA) were purchased from Sigma (St. Louis, MO, USA). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Janssen Chimica (Beerse, Belgium). *Trans*-parinaric acid (TPA) was from Molecular Probes (Eugene, OR, USA). Monopalmitoylphosphatidylcholine and egg lysophosphatidylcholine were obtained from Avanti Polar Lipids (Pelham, AL, USA). These and all other chemicals were of analytical grade. Double-distilled water was used.

2.2. Preparation of liposomes

Liposomes were prepared with the 'film' method. Appropriate mixtures of the phospholipids and/or cholesterol 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 and then hydrated with 50 mM acetate, Hepes or glycine buffer with 0.12 M sodium chloride to maintain the pH at 4.0, 7.4 or 10.5, respectively. Sometimes the liposome dispersions were downsized using an extrusion system (Sartorius, Göttingen, Germany) once through 0.6-μm- and three times through 0.2-μm-pore-size filters (Nuclepore, Cambridge, MA, USA), respectively. At the selected storage temperature the pH of the dispersion was measured and adjusted before and after extrusion, if necessary. The liposome dispersions were refrigerated overnight. Subsequently, the pH of the dispersion was measured again at the storage temperature and adjusted, if necessary.

2.3. Hydrolysis procedure

Ampoules were filled with the aqueous liposome dispersions under a nitrogen atmosphere and sealed. Ampoules were stored at 30 or 70°C in a water bath. Samples were taken after appropriate time intervals.

2.4. Analytical methods

Phospholipids were analyzed by HPLC as described earlier [12]. Samples for HPLC analysis were prepared by the Bligh and Dyer extraction [13]. Phospholipids were collected in the chloroform phase, and after dilution of the chloroform phase in methanol, 100-μl aliquots of the solution were directly injected into the column. The HPLC system consisted of a type 400 solvent delivery system (Kratos, Ramsey, NJ, USA), a Kontron sampler MSI 660 (Kontron AG, 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 aminophase column (25 cm × 4.6 mm, I.D., 5 μm particle size, Du Pont, 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/10 mM ammonium dihydrogen phosphate solution pH 4.8 (57:38:4, v/v) or (64:26:5, v/v), depending on column-to-column variations. The flow rate was 1.5 ml/min.

The concentrations of water-soluble glycerophospho compounds were estimated by phosphorus determination according to Fiske and SubbaRow [14] in the supernatant of a Bligh and Dyer extract [13] of the liposome dispersion.

To monitor the contents of the external aqueous phase of the liposomes, 1.5 ml samples were ultracentrifuged at 160 000 × g for 45 min. and 1.0 ml aliquots of the supernatant were analyzed chemically.

Turbidity of the liposomes was measured at 450 nm in a 1-mm-light-path cuvette on a Pye Unicam Pu 6800 UV/VIS spectrophotometer (Philips/Pye Unicam, Cambridge, UK) at ambient temperature, except for DMPC-liposomes which were measured at 20 ± 0.1°C after cooling in ice-water. If necessary, the multilamellar liposome dispersions were diluted in buffer just before the turbidity measurement. Turbidity of downsized dispersions was measured without any dilution. Turbidity of the liposomes at different temperatures was monitored at 450 nm in a 1-mm-light-path cuvette on a double-beam spectrophotometer (Lambda 5 UV/VIS spectrophotometer, Perkin-Elmer, Norwalk, CT, USA). The temperature of the samples in the cuvette was controlled with a Digital Controller C570-0701 (Perkin-Elmer) and measured by a digital thermometer. The turbidity at 450 nm depended on parameters such as (phospho)lipid composition, concentration, size and batch (results not shown; see also [15]). Therefore, to compare the different dispersions, only the relative turbidity (com-

pared to the turbidity of fresh liposomes of the same batch at ambient temperature) is shown in this study.

To gain information about the bilayer rigidity of 0.18-μm-DPPC/DPPG (10:1)-liposomes, steady-state fluorescence anisotropy of DPH in the liposomal bilayers was measured at different temperatures. Five microliters of $2 \cdot 10^{-4}$ M DPH, dissolved in tetrahydrofuran, was added to 50, 100 and 150 μl of 22 mM 'fresh' or 'aged' liposomes diluted in buffer (total volume 3 ml). This mixture was stabilized for 1 h in the dark at ambient temperature. The fluorescence anisotropy measurements were performed on a LS50 luminescence spectrometer (Perkin Elmer), 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 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 [16]. The temperature of the samples was controlled with an external water bath and measured in a cuvette with a digital thermometer.

^{31}P-NMR spectra were recorded at 121.5 MHz on a Gemini 300 Broadband High Resolution FT NMR System (Varian Associates, Palo Alto, CA, USA) at ambient temperature. A gated decoupling technique was used, with an acquisition time of 0.8 s, a relaxation delay of 2 s, a 3.3-μs pulse (90° pulse 10.1 μs), a spectral width of 30 kHz and 48 000 data points. Prior to Fourier transformation exponential multiplication was applied, resulting in a 50-Hz line broadening. The number of accumulated decays was 18 816 in the case of liposomes and 4704 in the case of a micellar solution. Zero ppm corresponds with the chemical shift position of phosphoric acid.

The Z-average particle size and polydispersity index (p.d.) were determined by dynamic light scattering (DLS) with a Malvern 4700 system using a 25-mW He-Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvern, 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 size distribution up to 1.0 for a completely heterogeneous one.

3. Results

The long-term chemical and physical stability of liposome dispersions differing in composition, charge, concentration, size and pH was examined. The percent hydrolysis of the liposome dispersions in this study was defined as

$$\%\text{hydrolysis} = 100\% - [\text{PC}] \times 100\%/([\text{PC}] + [\text{LPC}] + [\text{GPC}]) \quad (1)$$

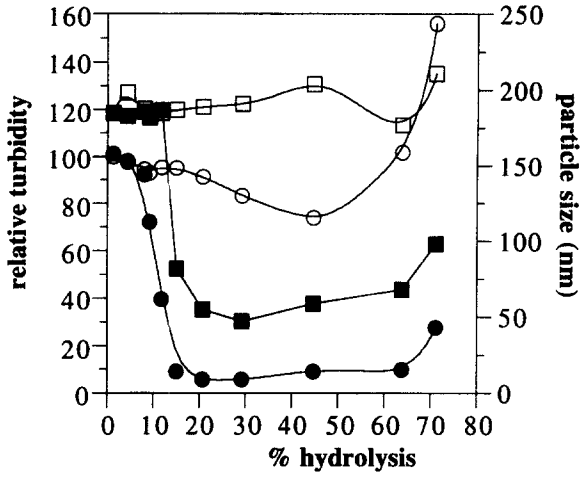

Fig. 1. Typical plots of relative turbidity at 450 nm and particle size (monitored by DLS) against percent hydrolysis of 0.18-μm DPPC/DPPG (10:1)-liposomes before and after heating for 1 min at 70°C. These liposomes were hydrolyzed at pH 4.0 and 30°C. ○: relative turbidity before heating; ●: relative turbidity after heating; □: size before heating; ■: size after heating.

where [PC] is the concentration of phosphatidylcholine, [LPC] is the concentration of lysophosphatidylcholine (the first hydrolysis product of PC) and [GPC] is the concentration of glycerophospho compounds (the end-products of PC hydrolysis) [17]. Except in the case that the liposomes were composed of 100% DPPG, the contribution of glycerophospho compounds generated by hydrolysis of PG was neglected.

Fig. 1 shows the relative turbidity at 450 nm and the particle size (determined by DLS) against the percent hydrolysis of 22 mM 0.18-μm DPPC/DPPG (10:1)-liposomes, stored at pH 4.0 and 30°C. Hardly any change in size was observed upon hydrolysis of these liposomes. Only above a hydrolysis percentage of 40% the particle size tended to grow and the p.d. of the dispersions increased (from 0.1 to 0.2–0.4). The relative turbidity at 450 nm of these liposomes was only minimally affected by the hydrolysis process as well (see Fig. 1). The gradual changes in the relative turbidity plot are probably due to chemical changes. The increase in turbidity and particle size above $\pm$40% hydrolysis indicated the beginning of the formation of aggregates with high fatty acid content as could be clearly visually observed upon prolonged storage. Fatty acids are the only lipophilic compounds that remain upon complete hydrolysis of PC and LPC and are known to form other types of aggregates than liposomes [18].

However, after heating for 1 min at 70°C in a water bath (temperature of sample > 65°C), major changes were observed in both particle size and turbidity upon cooling: both parameters decreased above a critical hydrolysis percentage of ca. 9%. The dependence of the relative turbidity on the temperature for 0.18-μm DPPC/DPPG (10:1)-liposomes with different levels of phospholipid degradation was also plotted in Figs. 2 and 3. At low levels of

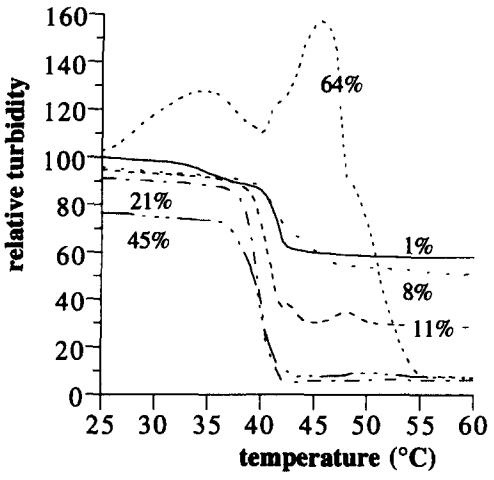

Fig. 2. Typical heating curves of relative turbidity at 450 nm against the temperature of 0.18-μm DPPC/DPPG (10:1)-liposomes hydrolyzed at pH 4.0 and 30°C. The percent hydrolysis of the samples are indicated in the figure.

hydrolysis a reversible change in turbidity of these liposomes was observed at the phase-transition temperature (41.5°C [19]) (see Fig. 3A). However, at higher levels of hydrolysis irreversible changes in turbidity of the DPPC/DPPG (10:1)-liposomes were observed when passing through the phase-transition temperature (see Fig. 3B and C). At hydrolysis levels just above the critical hydrolysis percentage, the fall in turbidity was not yet complete in one heating cycle yet. A further decrease in turbidity was found upon repeating the cooling and heating cycles (Fig. 3B). At higher hydrolysis levels the fall in turbidity was completed in only one cycle (Fig. 3C). The fall in turbidity could not be triggered by ultrasonic irradiation in a water bath for 1 min at ambient temperature (only a small decrease in turbidity was found which did not depend on the percent hydrolysis). The turbidity of 'collapsed' liposome dispersions remained stable upon storage at ambient temperature for at least two weeks.

For samples in which a drop in turbidity and particle size was found, the phosphorus concentration in the supernatant obtained after ultracentrifugation (see Section 2) increased to ±60% of the total amount. Hardly any differences were observed between the LPC/DPPC ratios of the total dispersion and the supernatant obtained after ultracentrifugation of hydrolyzed liposomes. This suggests that no demixing between DPPC and LPC had occurred.

The physical organization of the hydrolyzed 0.18-μm DPPC/DPPG (10:1)-liposomes before and after heating was also investigated by ^{31}P-NMR. The results are shown in Fig. 4. Fig. 4A shows a ^{31}P-NMR spectrum of freshly prepared 0.18-μm DPPC/DPPG (10:1)-liposomes at pH 4.0 (2% hydrolyzed). The broad, asymmetric shoulder peak indicates that all phospholipids were in a lamellar organization and part of an assembly with a low curvature [20,21]. After heating in a water bath at 70°C for 1 min, the same spectrum was observed (result not shown). The ^{31}P-NMR spectrum of 26% hydrolyzed 0.18-μm DPPC/DPPG (10:1)-liposomes (Fig. 4B) was almost identical to that in Fig. 4A. The only difference between Fig. 4A and B was a small symmetric peak above the shoulder peak at 0 ppm, indicating the presence of about 4%

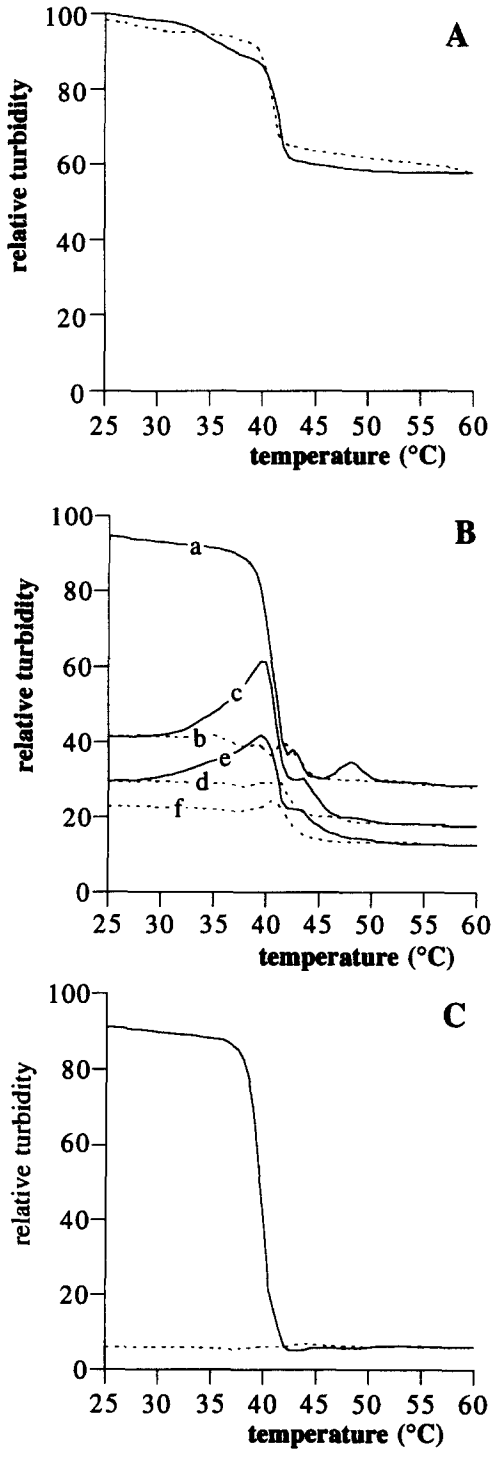

Fig. 3. Typical sequential heating (————) and cooling (- - -) curves showing the relative turbidity at 450 nm against the temperature of 0.18-μm DPPC/DPPG (10:1)-liposomes. The percent hydrolysis was 1% (A), 11% (B) and 21% (C). The letters a–f in B indicate the sequence of the heating and cooling curves.

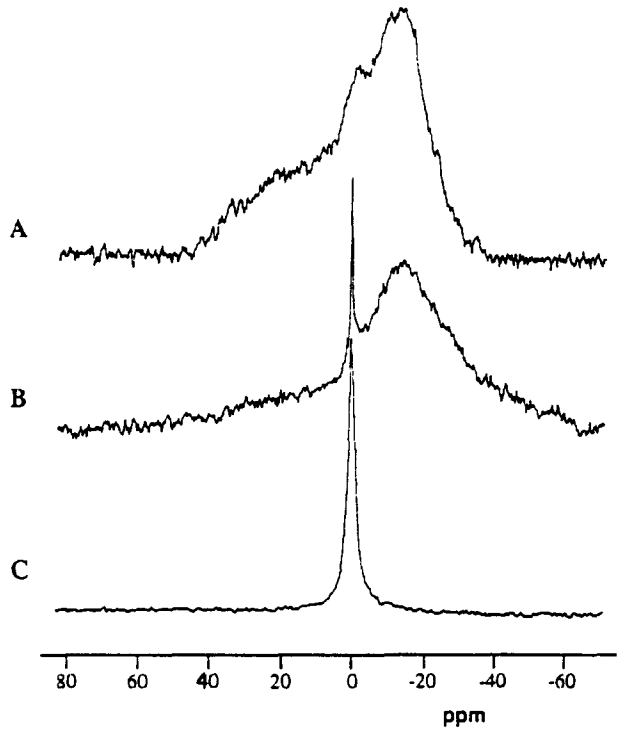

Fig. 4. ^{31}P-NMR spectra of 0.18-μm DPPC/DPPG (10:1)-liposomes hydrolyzed at pH 4.0 and 30°C before (A and B) and after (C) heating for 1 min at 70°C. The percent hydrolysis was 2% (A) and 26% (B and C). (A) $\Delta\nu_{1/2}$ = 26 ppm; $\Delta\nu_{1/4}$ = 52 ppm, $\Delta\nu_{1/8}$ = 68 ppm; asymmetry = 1.7 B: $\Delta\nu_{1/2}$ = 31 ppm, $\Delta\nu_{1/4}$ = 44 ppm, $\Delta\nu_{1/8}$ = 72 ppm; asymmetry = 1.1 C: $\Delta\nu_{1/2}$ = 2 ppm, $\Delta\nu_{1/4}$ = ppm, $\Delta\nu_{1/8}$ = 7 ppm; asymmetry = 0.4 $\Delta\nu_{1/2}$, $\Delta\nu_{1/4}$ and $\Delta\nu_{1/8}$ are the line widths in ppm measured at 1/2, 1/4 and 1/8 of the height of the chemical shift. Asymmetry is the ratio of the widths at half height of the left part over the right part of the vertical line through the maximum of the spectrum.

phosphorus compound in an isotropic phase. This small isotropic signal probably resulted from the water-soluble GPC (= 6 mol%). After heating this 26%-hydrolyzed liposome dispersion for 1 min at 70°C, a dramatic change was observed in the ^{31}P-NMR spectrum of this sample. Only one large, symmetric narrow peak was detectable, indicating that all phosphorus compounds were in an isotropic phase (Fig. 4C). This signal could be fully quenched by addition of a solution of manganese chloride (final concentration 5 mM). The presence of manganese chloride also caused an increase in turbidity. No attempt was made to further investigate the consequences of the increase in turbidity by manganese chloride for the outcome of the experiment.

The bilayer rigidity of 0.2-μm DPPC/DPPG (10:1)-liposomes as a function of percent hydrolysis was monitored by steady-state fluorescence anisotropy of incorporated DPH at different temperatures. The results are shown in Figs. 5–7. At ambient temperatures (below the main phase-transition temperature (T_m)) the anisotropy values are relatively high (about 0.35, see Fig. 5) indicating a rigid environment, typical for a gel phase ($L_{\beta'}$ phase). Upon heating, a relatively small decrease in anisotropy

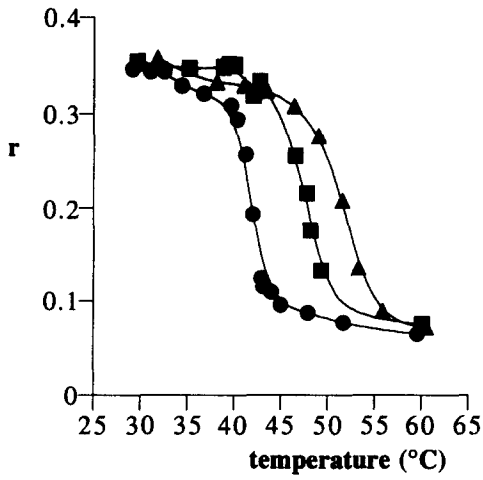

Fig. 5. Typical fluorescence anisotropy values (r) of DPH against the temperature of hydrolyzed 0.18-μm DPPC/DPPG (10:1)-liposomes as measured upon heating. These liposomes were hydrolyzed at pH 4.0 and 30°C until a hydrolysis percentage of 1% (●), 17% (■) or 63% (▲) was reached.

values was observed at about 35°C (= pre-transition temperature), indicating the so-called rippled gel phase ($P_{\beta'}$). Heating to a temperature above 40°C resulted in a pronounced decrease in anisotropy values. The low anisotropy values above 50°C (about 0.06) are characteristic of the liquid crystalline phase (L_α). Upon hydrolysis, the drop of DPH fluorescence anisotropy values in the heating curves shifted to higher temperatures (see Figs. 5 and 7), indicating that the T_m increased upon hydrolysis. The anisotropy values of DPH well above and below the main phase-transition did not change upon chemical hydrolysis and upon drop in turbidity. The decrease in anisotropy values covered a wider temperature range with increasing extent of hydrolysis (see Fig. 5). This indicates a broadening of the main phase-transition. Indications for the presence of $P_{\beta'}$

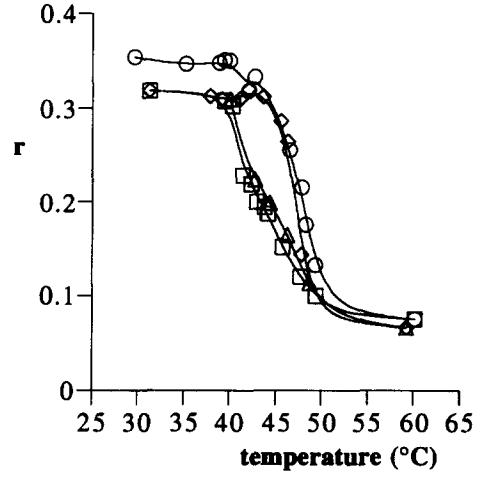

Fig. 6. Typical sequential cooling and heating curves of fluorescence anisotropy values (r) of DPH against the temperature of 0.18-μm DPPC/DPPG (10:1)-liposomes (17% hydrolyzed). ○: 1st heating curve; □: 1st cooling curve; ◇: 2nd heating curve; △: 2nd cooling curve.

disappeared upon hydrolysis; the small decrease of the anisotropy values around 35°C did not occur (see Fig. 5). At low levels of hydrolysis the anisotropy values measured during subsequent heating and cooling cycles were identical (result not shown). However, when the liposomes had a hydrolysis percentage above the critical hydrolysis percentage, hysteresis occurred and anisotropy values measured during a cooling cycle were not identical to the anisotropy values measured during a heating cycle; the cooling curves shifted to lower temperatures compared with the heating curves (see Fig. 6). The temperature values for the main phase-transition as derived from the cooling and heating curves are shown in Fig. 7. Fluorescence anisotropy measurements of hydrolyzed, 0.18-μm DPPC/DPPG (10:1)-liposomes were also performed with the probe *trans*-parinaric acid (TPA). TPA 'prefers' a rigid environment over a fluid environment [22]. Therefore, upon heating, the anisotropy values of TPA in a bilayer should only decrease if all bilayer domains are in a liquid crystalline phase. For DPH, which is distributed evenly over all bilayer domains, anisotropy values are expected to decrease more gradually. Large differences between anisotropy plots of both probes against the temperature would indicate the presence of different domains in the liposome bilayers. However, no such differences were observed between DPH and TPA anisotropy plots as a function of temperature with liposomes of different hydrolysis levels. Therefore, there is no indication for phase separation in the bilayers of 0.18-μm 'fresh' or 'aged' DPPC/DPPG (10:1)-liposomes upon hydrolysis.

Further experiments were performed to investigate if other liposome dispersions also showed the phenomena mentioned above. Variables of investigation were bilayer composition, concentration of phospholipids, size of the

Table 1
Results of relative turbidity and size measurements to monitor physical changes upon chemical hydrolysis [a]

Temperature (°C)	Liposome composition	Concentration (mM)	Sized through 0.2 μm pores	pH	k_{obs} (10^{-7} s^{-1}) [b]	Critical % hydrolysis [c]	Critical time (h) [c]
30	DMPC	20	no	4.0	1.5 ± 0.1	6 ± 1	110
	DPPC	4	no	4.0	1.3 ± 0.1	8 ± 1	180
	DPPC	20	no	4.0	1.2 ± 0.1	9 ± 1	220
	DPPC	100	no	4.0	1.3 ± 0.1	8 ± 3	180
	DPPC	200	no	4.0	1.1 ± 0.1	11 ± 1	290
	DSPC	20	no	4.0	1.2 ± 0.1	20 ± 2	520
	EPC	20	no	4.0	1.0 ± 0.1	no drop	no drop
	DPPC/DPPG (10:1)	22	no	4.0	2.4 ± 0.1	8 ± 1	100
	DPPC/DPPG (10:1)	22	yes	4.0	2.6 ± 0.2	8 ± 1	90
	DSPC/DPPG (10:1)	22	no	4.0	2.7 ± 0.1	15 ± 1	170
	DPPC/CHOL (10:4)	28	no	4.0	1.6 ± 0.2	no drop	no drop
	DPPC/DPPG/CHOL (10:1:4)	30	no	4.0	1.8 ± 0.1	no drop	no drop
	DPPG	20	no	4.0	19 ± 3 [d]	< 1	0
70	DMPC	20	no	4.0	16 ± 1	6 ± 1	11
	DPPC	20	no	4.0	18 ± 1	10 ± 1	16
	DPPC	20	no	7.4	5.9 ± 0.6	12 ± 1	60
	DPPC	20	no	10.5	41 ± 3	11 ± 1	8
	DSPC	20	no	4.0	15 ± 1	16 ± 2	32
	EPC	20	no	4.0	16 ± 2	no drop	no drop
	DPPC/DPPG (10:1)	22	yes	4.0	27 ± 4	8 ± 1	9
	DPPC/DPPG (10:1)	22	yes	7.4	3.8 ± 0.1	11 ± 1	85
	DPPC/DPPG/CHOL (10:1:4)	30	yes	4.0	21 ± 1	no drop	no drop
	DPPC/CHOL (10:4)	28	no	4.0	21 ± 1	no drop	no drop
	EPC/EPG (10:1)	22	yes	4.0	19 ± 1	no drop	no drop

[a] All experiments were done in triplicate. Liposomes were hydrolyzed at the indicated temperature and pH. Liposomes hydrolyzed at 30°C were heated for 1 min at 70°C before measurements. Turbidity and particle size measurement were performed at ambient temperatures. Typical results of these measurements are shown in Figs. 1, 8 and 9.

[b] Hydrolysis shows pseudo-first-order kinetics [3–7]: $\ln(100\% - \%\ \text{hydrolysis}) = -k_{obs} \times \text{time}$, where k_{obs} is the observed rate constant. The k_{obs} values presented here are similar to k_{obs} values determined for PC elsewhere [36].

[c] These critical values were determined on the basis of plots as shown in Figs. 1, 8 and 9. One line was drawn through the points before the drop and a tangent was drawn through the point of maximal change in turbidity. The intersection of these two lines was taken as the critical value. The critical time values have been calculated by using the equation shown at b. Critical values for percent hydrolysis and LPC/PC × 100% were almost identical up to around 20% hydrolysis (within 1%). Thus, up to 20% degradation, the detailed chemical composition of the liposome dispersions can be derived easily. Example given: Upon 20% degradation, the liposome composition is about 80 mol% PC, 20 mol% LPC, 20 mol% fatty acids and 0–1% mol% GPC. Upon further hydrolysis of PC, part of LPC will also degrade further into water soluble GPC, so that molar ratio of LPC/fatty acids < 1. When PG is mixed with PC, the hydrolysis rate of PG is only slightly faster than PC in that dispersion and therefore assumed to be equal.

[d] Pure DPPG-liposomes do not follow pseudo-first-order kinetics, probably due to loss of charge from the bilayers upon hydrolysis [36]. The k_{obs} of DPPG has therefore been derived from the data above 50% hydrolysis.

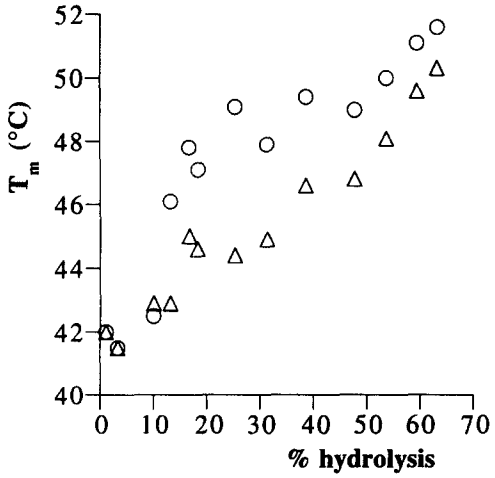

Fig. 7. The transition temperature (T_m) against percent hydrolysis of 0.18-μm DPPC/DPPG (10:1)-liposomes. These liposomes were hydrolyzed at pH 4.0 and 30°C. The T_m is assigned here to the midpoint of the range of maximum change in anisotropy of heating (○) and cooling curves (△) such as shown in Figs. 5 and 6.

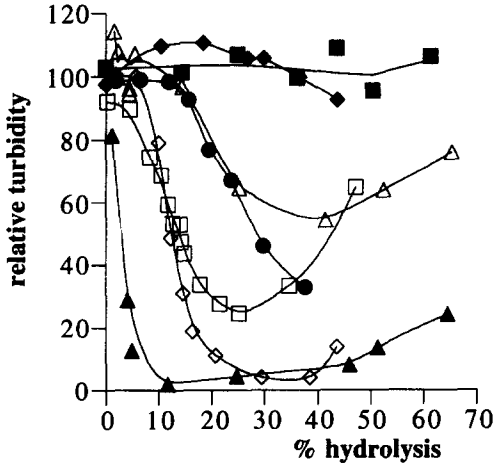

Fig. 9. Typical plots of the relative turbidity at 450 nm of liposomes against the percent hydrolysis after hydrolyzing at pH 4.0 and 30°C and after heating for 1 min at 70°C. The measurements were performed at ambient temperature. See Fig. 8 for the liposome compositions and their symbols.

liposomes, pH and storage temperature. The relative turbidity of these liposomes was measured at 450 nm and plotted against the percent hydrolysis. The results are shown in Figs. 8 and 9 and in Table 1. For liposomes extruded through 0.2-μm pores, the particle size of the liposomes was also measured by DLS (results not shown). The hydrolysis rate constants of the liposome dispersions and storage time to obtain certain levels of hydrolysis are also shown in Table 1.

In Fig. 8 the relative turbidity at 450 nm upon hydrolysis at pH 4.0 and 30°C is shown for non-downsized liposomes composed of DMPC, DPPC, DSPC, DPPC/CHOL (10:4), EPC, DPPC/DPPG (10:1),

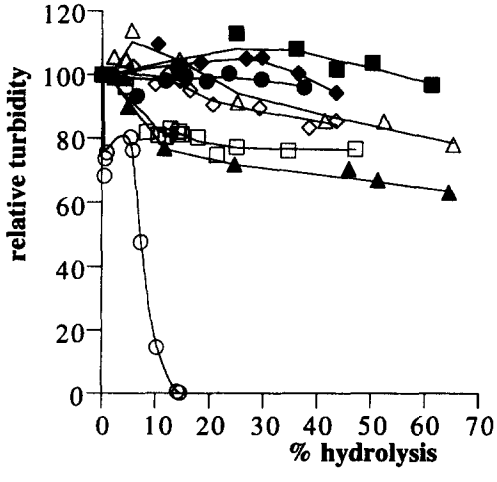

Fig. 8. Typical plots of the relative turbidity at 450 nm of liposomes against the percent hydrolysis after hydrolyzing at pH 4.0 and 30°C. The measurements were performed at ambient temperature, except the DMPC-liposomes which were measured at 20.0±0.1°C. The liposomes were composed of DMPC (○), DPPC (□), DPPC/DPPG 10:1 (◇), DSPC (△), DSPC/DPPG 10:1 (●), DPPC/CHOL 10:4 (■), EPC (◆) and DPPG (▲).

DSPC/DPPG (10:1) and DPPG. For these liposomes no drop was found in relative turbidity at 450 nm, except for DMPC-liposomes (clear solution already at 30°C). However, after heating in a water bath at 70°C for 1 min and cooling to ambient temperature, liposomes composed of DPPC, DSPC, DPPC/DPPG (10:1), DSPC/DPPG (10:1) and DPPG showed a drop in turbidity dependent on the level of hydrolysis (see Fig. 9). In particular, the results with DPPG-liposomes were striking. All freshly prepared DPPG-liposomes in acetate buffer (pH 4.0) showed a decrease in turbidity upon heating at 70°C immediately after preparation ($n = 9$). No precipitation was found in the case of DPPG-liposomes as might be expected when large amounts of calcium (which is used during the enzymatic transphosphatidylation to obtain DPPG) is present. For liposomes composed of DPPC/CHOL (10:4), DPPC/DPPG/CHOL (10:1:4) and EPC, the turbidity did not change significantly upon heating for 1 min at 70°C (see Fig. 9 and Table 1), even at high levels of hydrolysis (> 60%).

When liposome dispersions were stored at 70°C and pH 4.0, the same phenomena as described above were observed. No drop in turbidity occurred until a critical hydrolysis percentage was reached and the dispersion was cooled down below the major phase-transition range (see Table 1).

Apparently, the critical hydrolysis percentage and the maximal drop in relative turbidity also depended on the chain length of the liposomal phospholipids (critical hydrolysis percentage: DMPC < DSPC) and on the head group of the liposomal phospholipids (critical hydrolysis percentage: DPPG < DPPC) (see Figs. 8 and 9 and Table 1). This drop in turbidity of liposomes did not depend on phospholipid concentration (4–200 mM), pH (4.0, 7.4 and 10.5), storage temperature (30 and 70°C) and size (0.2 μm and

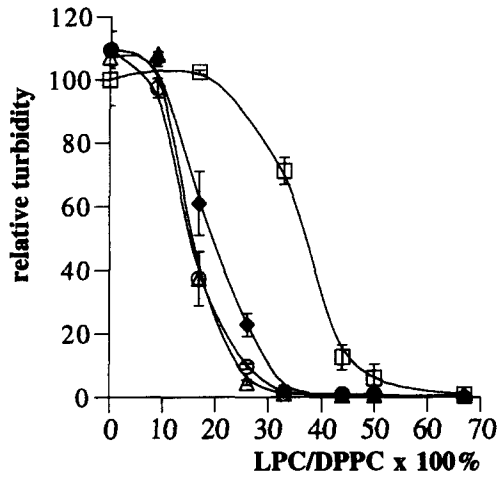

Fig. 10. The relative turbidity at 450 nm against the LPC/DPPC × 100% of liposomes composed of mixtures of DPPC, FA and LPC. Vertical bars indicate S.D. for three determinations. When no bars are shown, S.D. fell within symbol dimensions. The liposomes were composed of DPPC/LPC 100:*X* (□), DPPC/FA/LPC 100:10:*X* (◇), DPPC/FA/LPC 100:20:*X* (△), DPPC/FA/LPC 100:40:*X* (○) (*X* is *x*-coordinate).

non-downsized) of the liposomes (see Table 1). Adding 10% DPPG to DPPC- or DSPC-liposomes did not affect the critical hydrolysis percentage, but had a small effect on the maximum drop in relative turbidity (see Fig. 9 and Table 1).

Similar results (not shown) as those in Figs. 8 and 9 were obtained when the relative turbidity of liposomes at 450 nm was plotted against LPC/PC × 100%. Up to 20% degradation, the percent hydrolysis and the LPC/PC × 100% were almost identical (within 1%; data not shown). Thus, up to 20% degradation the detailed chemical composition of the liposome dispersions can be derived easily (see footnote c to Table 1).

Finally, 'mimics' of hydrolyzed liposomes were prepared to demonstrate the effect of the presence of palmitic acid (FA) and/or LPC on the relative turbidity of DPPC-liposome dispersions. These dispersions are hydrated above the phase-transition temperatures (70°C) and then cooled to ambient temperature. The results are shown in Fig. 10. Addition of LPC to DPPC-liposomes only resulted in a drop of relative turbidity above about 30 mol%. Addition of only FA to DPPC-liposomes (up to 40 mol%) hardly affected the relative turbidity. Interestingly, addition of both LPC and FA to DPPC-liposomes resulted in a synergistic effect: the drop in relative turbidity was already found above about 9% LPC/DPPC, a value similar to the one encountered when hydrolyzing DPPC-liposomes.

4. Discussion and conclusions

In this study, consequences of chemical hydrolysis for the physical stability of liposomes were studied. It has been reported before that chemical hydrolysis may lead to an increase in particle size [9] and to an increase in permeability of liposomal bilayers [10]. In this study, it is demonstrated for the first time that as a consequence of chemical hydrolysis and under specific conditions, phospholipid liposome dispersions show a pronounced drop in liposome size as indicated both by DLS and by measuring turbidity of the liposome dispersions. These specific conditions include (1) that the liposomes pass through a gel-to-liquid crystalline phase-transition during a heating or cooling scan and (2) that the chemical hydrolysis exceeds a critical percentage (or critical LPC/PC ratio). We demonstrated that this phenomenon occurred for liposomes composed of DMPC, DPPC, DSPC or DPPG, which have a T_m of 23.5, 41.5, 55.5 or 41.5°C, respectively [19]. Liposomes which do not have such a phase-transition in the experimental range (such as liposomes composed of unsaturated phospholipids (EPC and/or EPG) and liposomes containing high mole fractions of CHOL remain physically stable, as monitored by turbidity and particle size measurements, even for high levels of hydrolysis ($\geq$ 40%). Grit and Crommelin also did not find a change in particle size with liposomes composed of partially hydrogenated EPC, natural EPG and CHOL [10]. Even non-downsized and non-charged liposomes without a phase-transition in the experimental range remained physically stable upon storage, as could be derived from constant turbidity data (see Figs. 8 and 9). Aggregation, as reported by Petersen and Chan for sonicated DPPC-liposomes [23], was not found here.

The large decrease in average particle size and turbidity strongly suggests the formation of micellar products. This is supported by: (1) a large part of the dispersion in which a drop of turbidity and particle size was found remained in the supernatant after 45 min centrifugation at 160 000 × *g* (about 60% of the phospho compounds), while all the non-hydrolyzed liposomes precipitated, (2) the ^{31}P-NMR spectrum of a 'collapsed' liposome dispersion was identical to that of an aqueous dispersion of DPPC with an excess of LPC [24] and could be fully quenched by manganese chloride, which suggest complete exposure of phosphate groups to the external medium, and (3) the relative turbidity of hydrolyzed DPPC-liposomes and 'mimic dispersions' (which are known to form micelles at high LPC concentrations, see below) dropped at a similar value of LPC/DPPC × 100% (compare Figs. 1 and 9 with Fig. 10).

The decrease in turbidity of 0.18-μm DPPC/DPPG (10:1)-liposomes with low level of hydrolysis at higher temperature depends only on passing of the liposomes through the gel-to-liquid crystalline phase-transition (see the curves with 1 and 8% hydrolysis shown in Fig. 2 and the curves shown in Fig. 3A). In L_α the partial molar volume of the phospholipids are larger than in $L_{\beta'}$, which causes a decrease in refractive index and therefore turbidity [15]. However, the decrease of the DPPC/DPPG (10:1)-liposomes with hydrolysis levels above the threshold value showed a combined effect of the change in

refractive index and particle size (see the curves with hydrolysis degrees of $\geq 11\%$ in Figs. 2 and 3B,C). The changes in particle size are shown in Fig. 1.

Lysophospholipids are well known for their ability to destabilize phospholipid bilayers, resulting in the formation of micelles [20,24–26]. Similar plots to those shown in Fig. 10 have been reported before [24,25]. Also the stabilization by cholesterol of phospholipid bilayers containing high amount of LPC as found in this study (see Figs. 8 and 9 and Table 1) has been observed before [25]. However, no attention has ever been paid to a possible role for fatty acids in this destabilization process, to our knowledge. It is well established that the presence of fatty acids in liposomal bilayers increases the tendency of liposomes to aggregate or fuse [27,28], or decreases the permeability of liposomes in a liquid crystalline phase [10,29]. The role of FA in the destabilization process reported here in this study is surprising. The presence of FA counteracted the permeability-enhancing effect of LPC on PC bilayers [10]. Therefore, FA were expected to stabilize bilayers as e.g., cholesterol does [25]. Cholesterol forms bilayers upon mixing with LPC in a 1:1 molar ratio [30]. Moreover, Jain et al. made liposome dispersions of unsaturated LPC and FA in a 1:1 molar ratio [31]. Therefore, a stabilization rather than a destructive effect was expected. The data presented in this study (especially Fig. 10) suggest that the LPC/fatty acid (1:1)-complex obtained in the study of Jain et al. is only a metastable organization. The physically most stable organization for such complexes (micelles) will only be obtained upon passing the phase-transition temperature. The difference between the data of Jain et al. and ours is probably related to the specific saturated compositions used in the present study and to the irreversible lamellar-to-micellar transformation that occurred for the saturated lipids.

The measurements of the fluorescence anisotropy of the lipophilic probe DPH in 0.18-μm DPPC/DPPG (10:1)-liposomes demonstrated that bilayer rigidity below and above the phase-transition temperature did not change upon chemical hydrolysis and upon formation of micelles (see Figs. 5–7). Grit et al. [10] also found hardly any change in bilayer rigidity of liposomes composed of partially hydrogenated EPC and/or cholesterol (in different molar ratios). One might expect that formation of micelles will decrease the packing of the phospholipids. This should result in a decrease of the fluorescence anisotropy values. However, this was not found in the present study. Therefore, we hypothesize that the micelles are disk-shaped (like two stacked coins) and that by far the largest fraction of the lipid molecules still maintains a bilayer packing. Fluorescence anisotropy data also demonstrated that the phase-transition temperature range broadened and shifted towards higher temperatures upon chemical hydrolysis (see Figs. 5–7). Hydrolysis of DPPC/DPPG (10:1)-liposomes resulted in phospholipid bilayers composed of the phospholipids DPPC and DPPG and the hydrolysis products LPC, LPG and FA. The subsequent hydrolysis products, glycerophosphatidylcholine and glycerophosphatidylglycerol, are water soluble and will therefore not accumulate in the bilayers. Until now, no study on the effect of the incorporation of both fatty acids and lysophospholipids in DPPC-liposomes has ever been done. It has been reported that up to 40 mol% incorporation of 1-palmitoyl-*sn*-glycerol-3-phosphorylcholine in DPPC liposomes at pH 7.0, only the sharp main transition peak of DPPC is observed, while the pre-transition disappeared [32]. Incorporation of palmitic acid in DPPC-liposomes resulted in a pH-dependent increase of the T_m and a broadening and an increase in enthalpy for the main transition [33–35]. Again, the pre-transition was abolished [33,34]. Based on these studies we hypothesize that the increase in T_m as observed here is due to the presence of fatty acids, one of the hydrolysis products. These observations were made by fluorescence anisotropy measurements of hydrolyzed 0.18-μm DPPC/DPPG (10:1)-liposomes. We confirmed them in our differential scanning calorimetry (DSC) studies of hydrolyzed, non-downsized DPPC-liposomes [36].

Above the critical hydrolysis percentage, anisotropy values measured during a cooling cycle were not identical to the anisotropy values measured during a heating cycle; the cooling curves shifted to the left compared with the heating curves (see Fig. 6). Such a hysteresis phenomenon has been described before for bilayers composed of stearoylsphingomyelin by Estep et al. [37]. They hypothesized that the bilayers are in a liquid crystalline phase at elevated temperatures. Upon decreasing the temperature, the bilayers supercool as a liquid crystalline phase which undergoes a transition to a metastable gel phase at lower temperatures than for the temperatures of the gel-to-liquid crystalline phase-transition of the heating curves. Kinetic barriers are so large that the thermodynamically more stable equilibrium gel state is not formed during the measurements. Probably the same happened here. Estep et al. claimed that impurities might stabilize the metastable gel structure, which is not so ordered as the equilibrium gel state. In the present study, the liposome bilayers consisted of DPPC, DPPG, their lysophospholipids and fatty acids upon hydrolysis. However, the exact nature of a possible metastable gel structure is not known yet. The various options include interdigitation of lysophospholipids [38].

In particular, the results with liposomes composed only of DPPG were striking. At pH 4.0 we were not able to make 'fresh' DPPG-liposomes which did not show the temperature-dependent conversion as described above. For several reasons pure DPPG-liposomes have never been used for drug delivery, but they have been used a number of times for basic studies at an extreme pH of 1.5 [39,40]. It is possible that the phenomena observed in the present study influenced the outcome of earlier experiments, in particular when DSC was used, because DSC results also depend on the particle size and the bilayer organization of the liposomes.

The finding that the T_m of hydrolyzed liposomes increases and the major phase-transition range broadens may have implications for the formulation of so-called thermosensitive liposomes [41]. These liposomes are designed to leak at a temperature which is a few degrees higher than body temperature. Temperature-triggered leakage must occur at a temperature close to the T_m of the liposomes at specific (heated) places in vivo (e.g., at places with cancer cells). However, upon hydrolysis during storage the T_m and major phase-transition will change. Any change in main phase-transition will affect the pharmokinetic profile of the encapsulated drug; the liposomes will leak at other places than desired.

In conclusion, chemical hydrolysis of liposomes hardly affects the particle size of the liposomes as long as the vesicles do not pass through their gel-to-liquid crystalline phase-transition. However, when such a transition occurs and the chemical hydrolysis exceeds a critical hydrolysis percentage or the phospholipid bilayer contains more than critical amounts of lysophospholipids and fatty acids, the bilayer undergoes a lamellar-to-micellar transition. The micelles retain a thermotropic behavior similar to that of the vesicles. The presence of cholesterol, or not passing the gel-to-liquid phase-transition temperature range prevents this phenomenon.

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