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Effects of phospholipid hydrolysis on the aggregate structure in DPPC/DSPE-PEG$_{2000}$ liposome preparations after gel to liquid crystalline phase transition

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Abstract

Upon storage of phospholipid liposome samples, lysolipids, fatty acids, and glycerol-3-phosphatidylcholine are generated as a result of acid- or base-catalyzed hydrolysis. Accumulation of hydrolysis products in the liposome membrane can induce fusion, leakage, and structural transformations of the liposomes, which may be detrimental or beneficial to their performance depending on their applications as, e.g., drug delivery devices. We investigated in the present study the influence of phospholipid hydrolysis on the aggregate morphology of DPPC/DSPE-PEG$_{2000}$ liposomes after transition of the phospholipid membrane from the gel phase to liquid crystalline phase using high performance liquid chromatography (HPLC) in combination with static light scattering, dynamic light scattering, and cryo-transmission electron microscopy (cryo-TEM). The rates of DPPC hydrolysis in DPPC/DSPE-PEG$_{2000}$ liposomes were investigated at a pH of 2, 4, or 6.5 and temperatures of 22 °C or 4 °C. Results indicate that following phase transition, severe structural reorganizations occurred in liposome samples that were partially hydrolyzed in the gel phase. The most prominent effect was an increasing tendency of liposomes to disintegrate into membrane discs in accordance with an increasing degree of phospholipid hydrolysis. Complete disintegration occurred when DPPC concentrations had decreased by, in some cases, as little as 3.6%. After extensive phospholipid hydrolysis, liposomes and discs fused to form large bilayer sheets as well as other more complex bilayer structures apparently due to a decreased ratio of lysolipid to palmitic acid levels in the liposome membrane.

Keywords: Phospholipid hydrolysis; Disc formation; PEG-lipid; Lysolipid; Fatty acid

1. Introduction

In an acidic or basic aqueous environment, phospholipid molecules are subjected to hydrolysis following pseudo first-order kinetics [1]. Hydrolysis rates depend on temperature and pH with a minimum at a pH of 6.5 [1–3]. Hydrolysis products of 1,2-diacyl-*sn*-glycerol-3-phosphatidylcholine are initially lysolipid (1-acyl-*sn*-glycerol-3-phosphatidylcholine) and the fatty acid, which, upon further hydrolysis of the lysolipid leads to the generation of glycerol-3-phosphatidylcholine and glycerol-3-phosphoric acid [4,5]. Accumulation of hydrolysis products in the liposome membrane has been shown to alter the integrity of the bilayer and to induce leakage, fusion, and transformation of liposomes into alternative aggregate structures [6–11]. Thus, the rate and the degree of phospholipid hydrolysis are major factors determining the shelf-life and the performance of liposomes intended for pharmaceutical or biotechnical applications. Phospholipid hydrolysis is inclined to particularly affect cholesterol-free liposomes used for instance in thermosensitive liposome formulations [12–16].

In previous studies, we have shown evidence of membrane disc formation in dispersions of lysolipid-containing thermosensitive liposomes that were heated to temperatures greater

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than their gel to liquid-crystalline phase transition temperature (T_C) and cooled again to temperatures below it (T_C cycling). The disc formation process was shown to depend on the presence of lysolipids and polyethyleneglycol-conjugated lipids (PEG-lipids) in the liposome membrane and we suggested that accumulation of these components at grain boundaries and other packing defects in the liposome membrane facilitated the structural reorganization of the liposome membrane [17,18].

Based on these findings it appeared plausible that hydrolysis-generated accumulation of lysolipids in the membrane of, e.g., 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylcholine (DPPC) liposomes would increase the propensity for transformation of liposomes into discs. Support of this assumption stems from previous studies showing that co-dispersion of DPPC [7,8] or 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylcholine (DMPC) [9] and their respective hydrolysis products (fatty acids and lysolipids) produced disc-like aggregate structures. The presence of membrane discs has also been suggested in partially hydrolyzed DPPC and DMPC samples [7,8,11].

Since many current liposomal drug formulations contain PEG-lipids in order to extend their circulation time in vivo [19,20] and since the presence of PEG-lipids in the liposomal membrane increases the likelihood of disc formation [17] we investigated in the present study the influence of phospholipid hydrolysis on the aggregate morphology of DPPC/1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}) liposomes after heating to temperatures greater than their T_C. High performance liquid chromatography (HPLC) in combination with dynamic light scattering and cryo-transmission electron microscopy (cryo-TEM) was used to investigate the correlation between the degree of hydrolysis of DPPC/DSPE-PEG_{2000} liposomes and their tendency to transform into membrane discs. Static light scattering was employed to determine whether the disintegration of liposomes required a full T_C cycle or could also be achieved by heating or cooling liposomes once through their T_C.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylcholine (DPPC), 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylcholine (DMPC), 1-palmitoyl-2-hydroxy-*sn*-glycerol-3-phosphatidylcholine (MPPC) and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). ^{3}H-labeled cholesterylhexadecyl ether was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA). Citric acid was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All chemicals were of reagent grade and used without further purification.

2.2. Liposome preparation

DPPC/MPPC/DSPE-PEG_{2000} (molar ratios: 87:9:4 or 78:18:4), DPPC/DSPE-PEG_{2000} (molar ratio: 90:4), and DPPC/MPPC (molar ratio: 82:18), liposomes were prepared by the lipid film hydration method [21]. Some liposome preparations contained trace amounts of ^{3}H-labeled cholesterylhexadecyl ether, a non-metabolizable, non-exchangeable membrane marker to track total lipid concentrations. Dried lipid films were hydrated with 300 mM citrate buffer at a pH of 2, 4, or 6.5 for 1 h with intermediate vortex mixing at lipid concentrations of approximately 100 mg/ml or 8 mg/ml at a temperature of 50 °C. Liposomes were extruded ten times through two stacked polycarbonate membranes filters (Whatman Inc. Nucleopore, Newton, MA) with a pore size of 100 nm at a temperature of 50 °C using either a thermobarrel extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada) attached to a circulating water bath or a mini-extruder (Avanti Polar Lipids Inc., Alabaster, AL) mounted on a heating block. Liposomes were never cooled to temperatures below their T_C from the start of the hydration process to the end of the extrusion process.

2.3. Particle size determination

DPPC/DSPE-PEG_{2000} liposomes (molar ratio: 90:4), were stored in the gel-phase in 300 mM citrate buffer at two of the most commonly used storage temperatures, room temperature and fridge temperature, i.e., 22 °C or 4 °C and a pH of 2, 4, or 6.5 at lipid concentrations of 98 mg/ml (119 μmol/ml), 100 mg/ml (122 μmol/ml), or 81 mg/ml (99 μmol/ml), respectively. The pH stability of all samples was confirmed once a month. At regular time intervals, aliquots of liposome samples were diluted to a lipid concentration of approximately 2 mg/ml with 0.9% saline. The particle size distribution in the sample was analyzed by dynamic light scattering using a Nicomp 270 fixed-angle submicron particle sizer (Pacific Scientific, Santa Barbara, CA) for 10 min at a count rate of approximately 350 kHz, a temperature of 22 °C, and a wavelength of 633 nm. Nanosphere™ (Duke Scientific Corporation, Paulo Alto, CA) with an average size of 92±3.7 nm was used as a particle size standard. The average particle size was determined by volume-weighted Gaussian analysis before and after heating aliquots of stored liposome samples for 10 min to temperatures above the T_C of the formulation, i.e., to 45 °C for liposomes prepared and stored at a pH of 4 or 6.5 or to 50 °C for samples prepared and stored at a pH of 2. The higher temperature for liposomes prepared and stored at a pH of 2 was chosen in order to heat these liposome samples above their T_C, which was determined by differential scanning calorimetry on a Pyris I DSC apparatus (Perkin Elmer, Norwalk, CT) at a scan rate of 10 °C/min to be slightly higher (46.7±0.1 °C, n=3) than that of liposomes prepared at a pH of 4 (42.5±0.3 °C, n=4) or at a pH of 6.5 (43.3±0.1 °C, n=3). However, after one T_C cycle, the apparent mean average particle size of liposomes prepared and stored at a pH of 2 that were heated to a temperature of 45 °C was not significantly different from liposomes that were heated to a temperature of 50 °C. Heating aliquots of liposome samples to temperatures above their T_C with subsequent cooling to room temperature will hereafter be denoted as one T_C cycle.

2.4. HPLC assay

From liposome samples described above, aliquots at a volume of 50 μl were withdrawn at regular time intervals for up to 7 months and stored at a temperature of −20 °C until further analysis. To determine MPPC concentrations, aliquots were dissolved in 950 μl of methanol/chloroform (9:1 vol/vol) and 100 μl of this solution was analyzed by HPLC. To determine DPPC concentrations, aliquots were dissolved in 950 μl of methanol/chloroform (9:1 vol/vol). To 200 μl of this, solution 1 ml of a DMPC solution at a concentration of 1.5 mg/ml in methanol/chloroform (9:1 vol/vol) was added as an internal standard and 20 μl of the mixture were analyzed by HPLC. Lipids were separated at a temperature of 30 °C on a Waters 2690 separation module equipped with a Symmetry C8 (4.6×250 mm i.d.) reverse phase column (Waters Co., Milford, MS). The column was eluted with 100% methanol as the mobile phase at a flow rate of 1 ml/min. Phospholipids were detected on a SEDEX Model 75 evaporative light scattering detector (S.E.D.E.R.E., Alfortville Cedex, France) at a temperature of 50 °C and a nitrogen pressure of 3.5 bar in the evaporation chamber. Chromatography peaks were identified by comparison of their retention times with those of authentic standards. Analyte concentrations were determined by using calibration curves of authentic standards. Observed hydrolysis rates (k_{obs}) were calculated from the slopes of plots of the ln-transformed DPPC concentrations versus time according to the integrated rate equation: $\ln[DPPC_t] = \ln[DPPC_o] - k_{obs}\,t$ with $[DPPC_o]$ as the initial DPPC concentration and $[DPPC_t]$ as the DPPC concentration at time t.

2.5. Cryo-TEM

Cryo-TEM images of liposome samples were obtained on a Zeiss EM 920 A transmission electron microscope (Carl Zeiss Inc., Oberkochen, Germany) as

described in detail elsewhere [22–25]. All samples were prepared at a temperature of 25 °C. Images were taken in zero-loss bright field mode with an acceleration voltage of 80 kV. Image sections were carefully chosen to represent a typical impression of all sample images.

2.6. Static light scattering

DPPC/MPPC/DSPE-PEG$_{2000}$ liposomes (molar ratios: 87:9:4 or 78:18:4), DPPC/DSPE-PEG$_{2000}$ liposomes (molar ratio: 90:4), and DPPC/MPPC liposomes (molar ratio: 82:18) were prepared at a concentration of 10 mM and a temperature of 50 °C in 300 mM citrate buffer, pH 6.5. Samples were diluted to a final lipid concentration of 0.5 mM in cuvettes preheated and maintained at a temperature of 48 °C. Static light scattering measurements were performed using a SPEX-fluorolog 1650 0.22 double spectrometer (SPEX Industries Inc, Edison, NJ) in right angle mode at a wavelength of 350 nm. Measurements were repeated after cooling to a temperature of 25 °C. A sample of DPPC/DSPE-PEG$_{2000}$ liposomes (molar ratio: 90:4) prepared in citrate buffer, pH 4, and stored at a temperature of 22 °C for 10 days was measured consecutively at temperatures of 25 °C, 48 °C, and 25 °C.

3. Results

3.1. Dependence of DPPC hydrolysis on storage time, temperature, and pH

When DPPC/DSPE-PEG$_{2000}$ liposomes were stored at a pH of 6.5 and temperatures of 22 °C or 4 °C, the DPPC concentrations stayed within 10% of the initial value of 115 μmol/ml during the investigated time period of 27 weeks (Fig. 1A). Hydrolysis rate constants could not be calculated because no consistent decrease in DPPC concentrations with time could be observed. When samples were stored at temperatures of 22 °C or 4 °C and a pH of 4 or 2, DPPC concentrations decreased according to pseudo first-order kinetics ($R^2 > 0.95$). Hydrolysis rate constants decreased with lower temperature and higher pH of the storage buffer (Table 1).

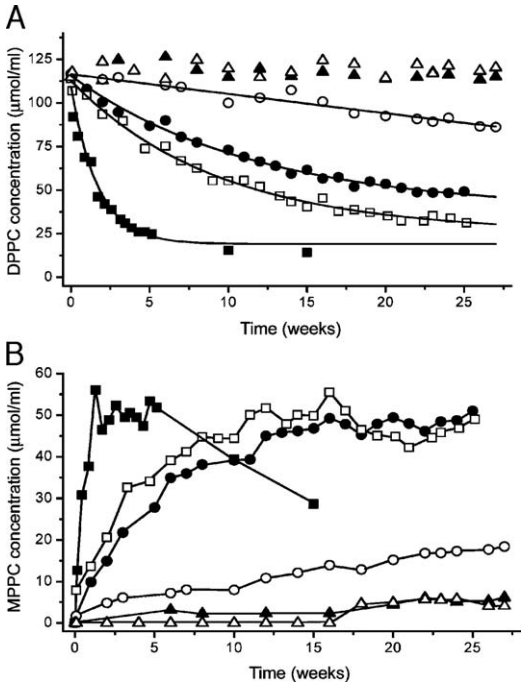

Fig. 1. Concentrations of (A) DPPC and (B) MPPC in DPPC/DSPE-PEG$_{2000}$ liposomes stored at temperatures of 22 °C (solid symbols) or 4 °C (open symbols) in 300 mM citrate buffer at a pH of 2 (■□) 4 (●○), or 6.5 (▲△). Lines in panel A were generated by fitting data to a first-order exponential decay.

Time-dependent changes in MPPC concentrations are illustrated in Fig. 1B. MPPC concentrations in samples prepared and stored at a pH of 6.5 and temperatures of 4 °C or 22 °C were close to or below the limit of detection at time points earlier than 18 weeks. At time points later than 18 weeks, mean MPPC concentrations in both samples were 5 μmol/ml. In liposome samples prepared and stored at pH 4 and a temperature of 4 °C, MPPC concentrations increased linearly ($R^2 = 0.98$) to 18.4 μmol/ml at 27 weeks. MPPC concentrations in liposome samples prepared and stored at a pH of 4 and a temperature of 22 °C increased from an initial value of 1.7 μmol/ml during the first 12 weeks asymptotically to a plateau value of approximately 50 μmol/ml. A similar asymptotic increase in MPPC concentrations was observed for liposomes prepared and stored at a pH of 2 and a temperature of 4 °C. The initial MPPC concentration in this preparation was 7.9 μmol/ml. In liposomes prepared at a pH of 2 and a temperature of 22 °C plateau values of approximately 50 μmol/ml were reached at 9 days and values remained at this level until at least 5 weeks. At 10 weeks and 15 weeks, MPPC concentrations decreased to 39 μmol/ml and 29 μmol/ml, respectively.

3.2. Effects of storage time, temperature, and pH on aggregate structure

Before the T_C cycle, the apparent[2] mean particle size of DPPC/DSPE-PEG$_{2000}$ liposomes stored at a pH of 6.5, 4, or 2 and temperatures of 22 °C or 4 °C was 100 nm and remained within 10% of this value over the investigated time period of 22 weeks. Only when liposomes were stored at a pH of 2 and a temperature of 22 °C for 28 days did the apparent mean particle size increase to 142 nm before the T_C cycle.

When liposomes were stored at a pH of 6.5 at temperatures of 22 °C or 4 °C and cycled through their T_C the initial apparent mean particle size in both samples was 94 nm, which did not change over the investigated time period of 22 weeks (Fig. 2). Cryo-TEM images revealed that gel-phase liposomes were polygonal in shape and their morphology did not change upon T_C cycling (Fig. 3). Membrane discs were largely absent in images of samples stored at both temperatures even after 33 weeks of storage at a temperature of 22 °C (Fig. 3C).

When liposomes were stored at a pH of 4 and a temperature of 22 °C the apparent mean particle size after one T_C cycle decreased gradually from 94 nm to 32 nm at 2 weeks. From that point onward, the apparent mean particle size increased until the

[2] The model used to calculate the average particle size from QELS data assumes the presence of spherical particles and the hydrodynamic radius is obtained from diffusion coefficient values according to the Stoke–Einstein equation. Hence, the mean particle size values determined for samples containing discs and other non-spherical aggregates may not be an accurate measure. A change in the mean particle size may, however, be used as an indication of major changes in the aggregate structure.

Table 1
Hydrolysis rate constants (k_{obs}) in DPPC/DSPE-PEG$_{2000}$ liposomes after storage at temperatures of 22 °C or 4 °C in 300 mM citrate buffer at a pH of 2 or 4

Storage temperature	$k_{obs} \times 10^{-7}$ (s^{-1}) pH of storage buffer	
	2	4
22 °C	4.95	0.59
4 °C	0.85	0.18

Hydrolysis rates were calculated from the slopes of plots of the ln-transformed DPPC concentrations versus time as presented in Fig. 1.

end of the investigated time period of 22 weeks (Fig. 2A). Storing liposomes at a temperature of 4 °C delayed the time point at which the apparent mean particle size minimum after T_C cycling (32 nm) could be observed to 20 weeks. At 22 weeks of storage, the apparent mean particle size increased again (Fig. 2B). Cryo-TEM analysis confirmed that before the T_C cycle, liposomes remained largely intact after 10 days of storage at a pH of 4 and a temperature of 22 °C (Fig. 4A). When liposomes were stored under these conditions for 7 days and cycled through their T_C, a considerable number of discs were formed and liposomes disintegrated completely into discs upon T_C cycling after 10 days of storage (Fig. 4B). When liposomes were stored at a pH of 4 and a temperature of 4 °C for 5 weeks some discs were initially present but a significant number of liposomes remained largely intact after the T_C cycle (Fig. 4C). When liposomes were stored under these conditions for almost 10 weeks, liposomes disintegrated completely into discs after one T_C cycle (Fig. 4D).

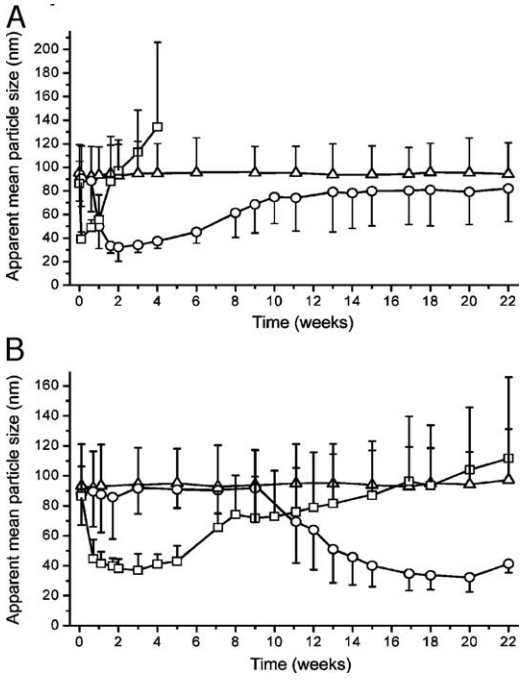

Fig. 2. Apparent mean particle size of DPPC/DSPE-PEG$_{2000}$ (molar ratio: 90:4) liposome preparations stored at a temperature of (A) 22 °C or (B) 4 °C at a pH of 2 (□), 4 (○), or 6.5 (△) and cycled one time through the T_C of the formulation. Error bars indicate the standard deviation of particle size determinations according to a count rate of approximately 350 kHz. Samples were analyzed for approximately 10 min.

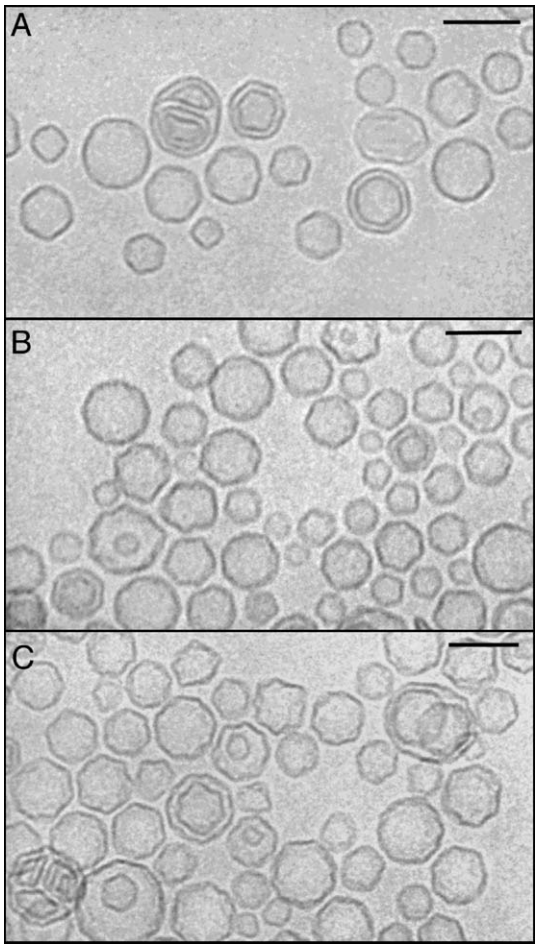

Fig. 3. Representative cryo-TEM images of DPPC/DSPE-PEG$_{2000}$ (molar ratio: 90:4) liposome stored at temperatures of 22 °C and a pH of 6.5. (A) Liposomes stored for 4 weeks before the T_C cycle. (B) The same sample as in panel A after one T_C cycle. (C) Liposomes stored for 33 weeks after one T_C cycle. Scale bars indicate a size of 100 nm.

When liposomes were stored at a pH of 2 and a temperature of 22 °C the apparent mean particle size after the T_C cycle decreased from 86 nm to a minimum of 39 nm after 1 day of storage. Upon further storage the apparent mean particle size increased continuously to an average size of 134 nm at 4 weeks (Fig. 2A). Since the sample became viscous at 3 weeks of storage, aliquots were no longer analyzed at time points later than 4 weeks. Storing liposomes at a temperature of 4 °C again delayed the time period at which changes in the apparent mean particle sizes after T_C cycling could be observed. In this case, a minimum in the apparent mean particle size (37 nm) could be observed between 1 and 5 weeks. From then on, the apparent mean particle size increased again until the end of the investigated time period of 22 weeks (Fig. 2B). Cryo-TEM images of freshly prepared and 5 day old liposome samples revealed a large number of membrane discs together with open liposome structures and intact liposomes before T_C cycling (Fig. 5A and E). After T_C cycling, the freshly prepared sample contained more discs with an average size comparable to the diameter of the liposomes (results not shown). After 1 day of storage under these conditions, liposomes disintegrated completely into small discs

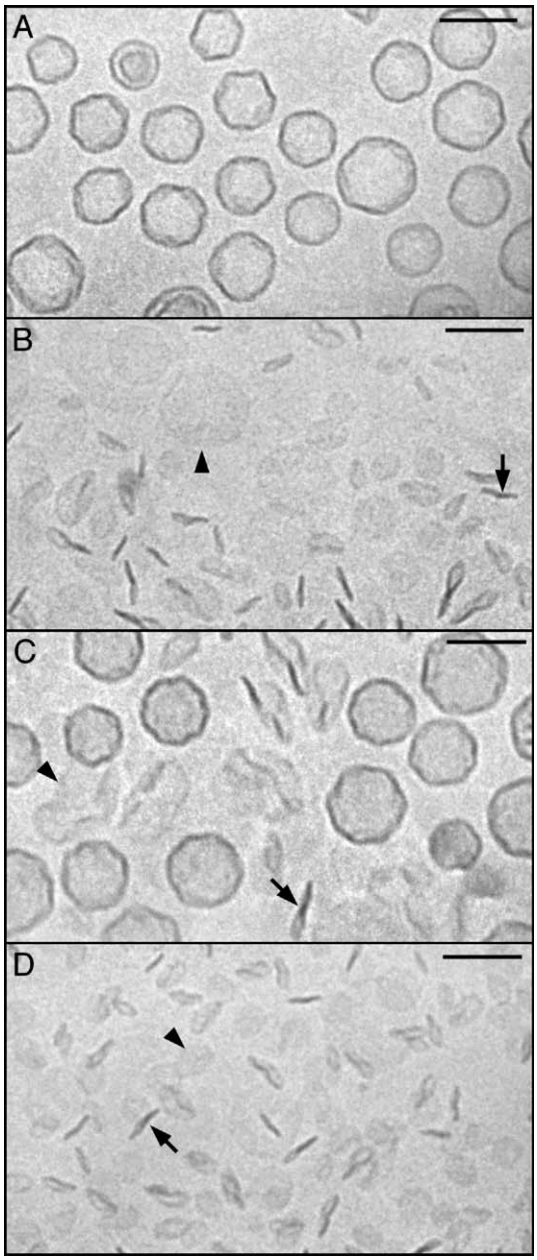

Fig. 4. Representative cryo-TEM images of DPPC/DSPE-PEG_{2000} (molar ratio: 90:4) liposomes stored at temperatures of 22 °C or 4 °C and a pH of 4. (A) Liposomes stored for 10 days at a temperature of 22 °C before the T_C cycle. (B) The same sample as in panel A after one T_C cycle. (C) Liposomes stored for 35 days at a temperature of 4 °C after one T_C cycle. (D) Liposomes stored for 67 days at a temperature of 4 °C after one T_C cycle. Black arrowheads indicate discs positioned face-on, black arrows indicate discs positioned edge-on. Scale bars indicate a size of 100 nm.

(Fig. 5B). When stored for 3 weeks, liposomes were no longer polygonal but instead spherical in shape before T_C cycling and the sample contained membrane discs as well as bilayer sheets (results not shown). After the T_C cycle, large bilayer sheets and large membrane discs were present in the sample (Fig. 5C). Upon further storage, bilayers, large liposomes, and large clusters of partially fused liposomes could be observed before the T_C cycle (Fig. 5D). When liposomes were prepared and stored at a pH of 2 and a temperature of 4 °C for 5 days, membrane discs coexisted with intact liposomes before T_C cycling (Fig. 5E). After T_C cycling, liposomes disintegrated completely into discs (Fig. 5F).

3.3. Evaluation of liposome disintegration during the gel to liquid-crystalline phase transition

Because the experimental set-up of particle size determination and cryo-TEM analysis did not allow measurements at high temperatures, these techniques could not be used to investigate whether disc formation required a T_C cycle or could also be achieved by transition from the gel to the liquid-crystalline phase. We therefore employed static light scattering, which allowed measurements at temperatures greater than the T_C of the liposome formulation.

As shown in Fig. 6, the mean light scattering intensity of freshly prepared DPPC/DSPE-PEG_{2000} liposomes (molar ratio: 90:4) increased when the temperature was decreased from values above the T_C of the formulation (48 °C) to temperatures below it (25 °C). This increase is not reflecting a change in the mean particle size but is instead a result of the larger partial molar volume of phospholipids in the liquid-crystalline phase resulting in a decreased refractive index [7 and references therein]. When the sample was heated again to a temperature of 48 °C, the mean light scattering intensity decreased by 46% to a value close to that of the original sample. When cooled again to 25 °C, the mean light scattering intensity increased back to a value close to that after the first cooling cycle. When liposomes were stored for 10 days at a temperature of 22 °C and a pH of 4, increasing the temperature to 48 °C lead to a decrease in the light scattering intensity by almost 80%. This decrease was 34% greater in comparison to that observed in the freshly prepared sample, strongly indicating the disintegration of liposomes into smaller aggregates, such as bilayer discs. As expected, cooling the stored sample back to a temperature of 25 °C increased the mean light scattering intensity only marginally.

3.4. Effects of PEG-lipids and lysolipids on aggregate structure

DPPC/DSPE-PEG_{2000} liposomes (molar ratio: 90:4), DPPC/MPPC liposomes (molar ratio: 82:18) and DPPC/MPPC/DSPE-PEG_{2000} liposomes (molar ratios: 87:9:4 or 78:18:4) were examined by static light scattering (Fig. 7) and cryo-TEM analysis (Fig. 8). These experiments served to investigate more specifically the influence of the presence of PEG-lipids and the percentage of lysolipids in the liposome membrane on the disc formation propensity after phase transition from the liquid-crystalline phase to the gel phase or upon T_C cycling.

Mean light scattering intensities were similar in all investigated liposome preparations when prepared and maintained at a temperature of 48 °C. When liposomes were cooled to a temperature of 25 °C, mean light scattering intensities increased in all samples except for DPPC/MPPC/DSPE-PEG_{2000} liposomes (molar ratio: 78:18:4) in which the mean light scattering intensity instead decreased (Fig. 7). Cryo-TEM analysis at a temperature of 25 °C revealed that DPPC/MPPC/DSPE-PEG_{2000}

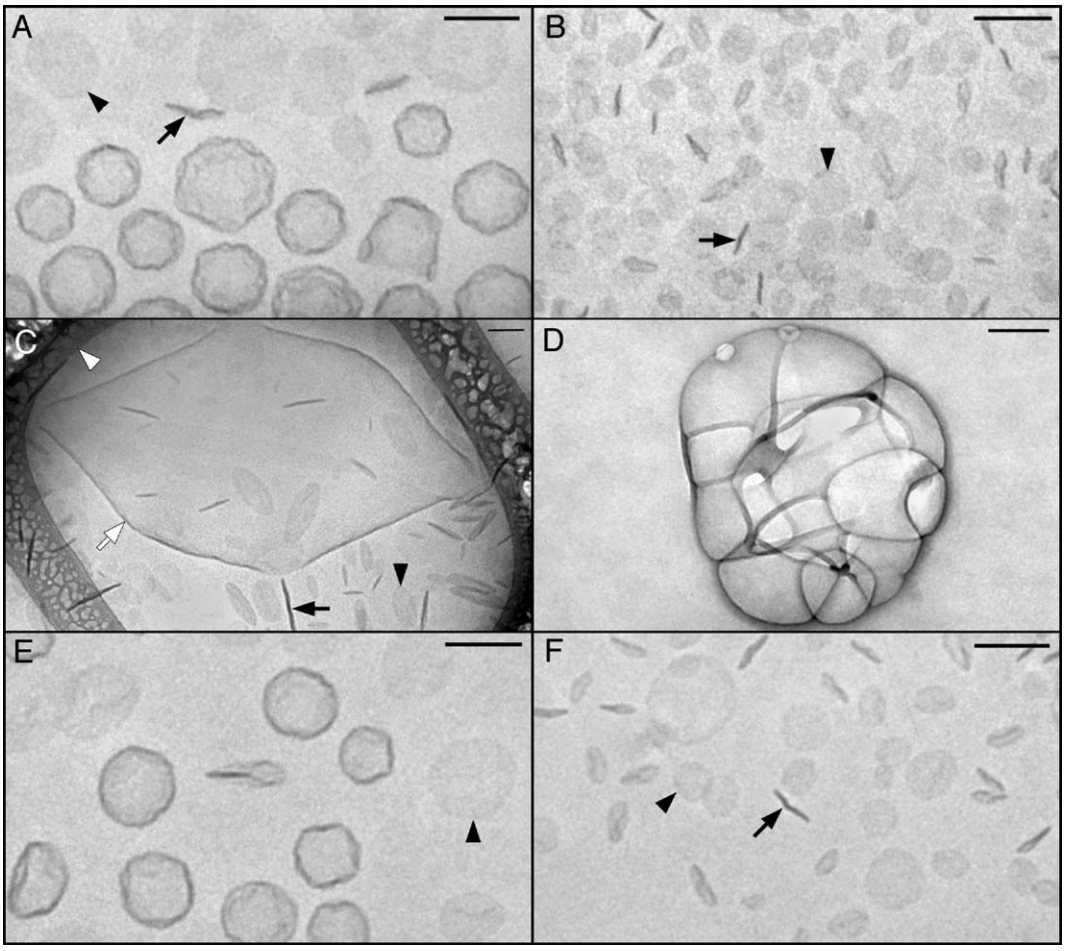

Fig. 5. Representative cryo-TEM images of DPPC/DSPE-PEG$_{2000}$ (molar ratio: 90:4) liposomes stored at a temperature of 22 °C or 4 °C and a pH of 2. (A) Sample the day of preparation before the T_C cycle. (B) Sample stored for 1 day at a temperature of 22 °C after one T_C cycle. (C) Sample stored for 21 days at a temperature of 22 °C after one T_C cycle. (D) Sample stored for 49 days at a temperature of 22 °C before the T_C cycle. (E) Sample stored for 5 days at a temperature of 4 °C before one T_C cycle. (F) The same sample as in panel E after one T_C cycle. Black arrowheads indicate discs positioned face-on, black arrows indicate discs positioned edge-on, white arrowheads indicate the polymer film, and the white arrow indicates a bilayer sheet. Scale bars indicate a size of 100 nm (note the different magnification in panel C.

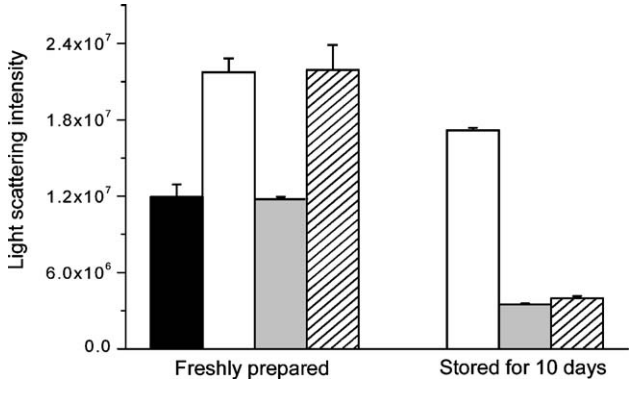

Fig. 6. Light scattering intensities of DPPC/DSPE-PEG$_{2000}$ (molar ratio: 90:4) liposomes at the day of preparation and after storage for 10 days at a temperature of 22 °C and a pH of 4. Light scattering intensities were determined in the sample that was never cooled to temperatures below 48 °C (black bars, $n=4$), cooled to a temperature of 25 °C (white bars, $n=4$), heated again to 48 °C (grey bars, $n=2$), and after cooling back to 25 °C (hatched bars, $n=2$). Error bars indicate the standard deviation.

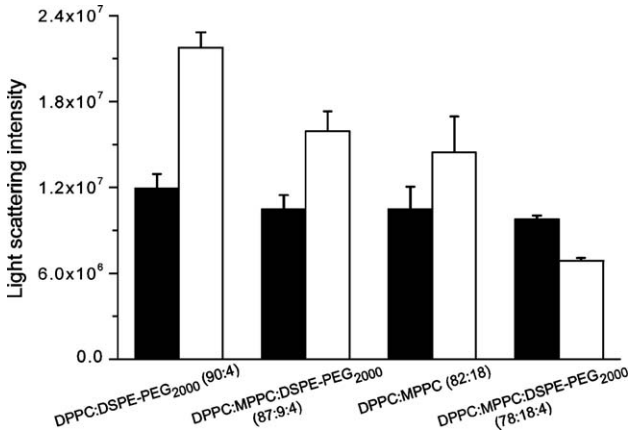

Fig. 7. Light scattering intensities of DPPC/DSPE-PEG$_{2000}$ (molar ratio: 90:4, $n=4$), DPPC/MPPC/DSPE-PEG$_{2000}$ (molar ratio: 87:9:4, $n=4$), DPPC/MPPC (molar ratio: 82:18, $n=2$) and DPPC/MPPC/DSPE-PEG$_{2000}$ (molar ratio: 78:18:4, $n=2$) liposomes that were prepared at a temperature of 50 °C and never cooled below 48 °C (black bars) or subsequently equilibrated to 25 °C (white bars). Error bars indicate the standard deviation.

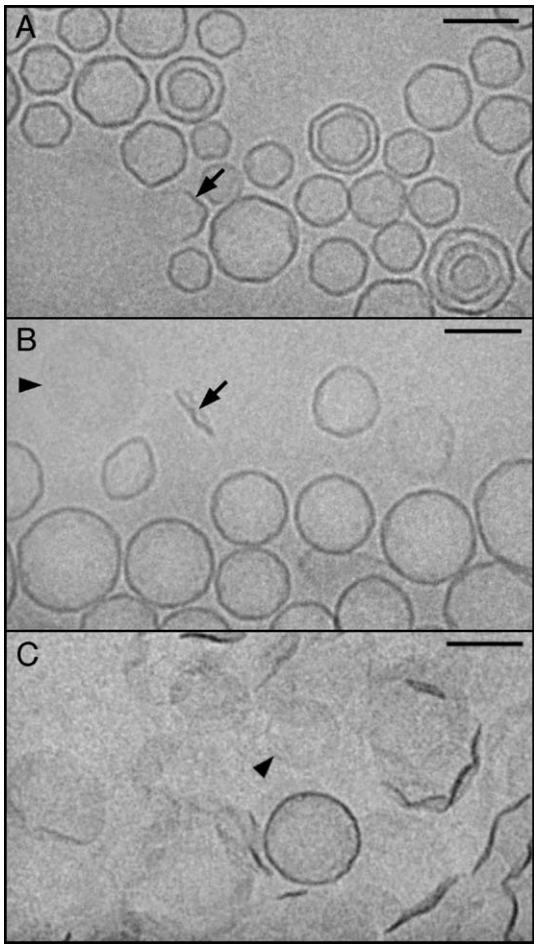

Fig. 8. Representative cryo-TEM images of (A) DPPC/MPPC/DSPE-PEG_{2000} (molar ratio: 87:9:4), (B) DPPC/MPPC (molar ratio: 82:18), and (C) DPPC/MPPC/DSPE-PEG_{2000} (molar ratio: 78:18:4) at a temperature of 25 °C. Liposomes were prepared at a temperature of 50 °C and a pH of 6.5. Scale bars indicate a size of 100 nm. Black arrowheads indicate discs positioned face-on, black arrows indicate discs positioned edge-on.

liposomes (molar ratio: 87:9:4) were polygonal in shape and discs were largely absent in the sample (Fig. 8A). After T_C cycling a small number of discs could be observed in the preparation (data not shown). Increasing the membrane content of MPPC to 18 mol% in the absence of PEG-lipids resulted in liposomes with a more spherical appearance and the presence of a small amount of membrane discs (Fig. 8B). In DPPC/MPPC/DSPE-PEG_{2000} liposomes (molar ratio: 78:18:4), membrane discs and open liposomes were the predominant aggregates. A small number of intact liposomes were also present in the sample (Fig. 8C). After T_C cycling, the disc content in DPPC/MPPC liposomes (molar ratio: 82:18) and DPPC/MPPC/DSPE-PEG_{2000} liposomes (molar ratio: 78:18:4) was not different (data not shown). Cryo-TEM results were consistent with results obtained by static light scattering experiments and confirm that the decrease in the mean light scattering intensity of DPPC/MPPC/DSPE-PEG_{2000} liposomes (molar ratio: 78:18:4) indicates the disintegration of liposomes into bilayer discs. Similar results were obtained for liposome formulations containing MSPC instead of MPPC (data not shown).

4. Discussion

4.1. Correlation between disc formation and the degree of hydrolysis

We demonstrated in the present study that the hydrolysis rate of DPPC/DSPE-PEG_{2000} liposomes is pH and temperature dependent (Table 1) and follows pseudo first-order kinetics in agreement with previous reports by other research groups on pure DPPC liposomes [1–3]. Hydrolysis rate constants were also comparable to those reported previously for DPPC liposomes under similar experimental conditions [1]. The degree of phospholipid hydrolysis was strongly correlated to the extent of disc formation induced by heating liposomes through their T_C.

According to cryo-TEM analysis, complete disintegration of liposomes into discs upon heating to temperatures greater than their T_C occurred at approximately 10 days of storage at a pH of 4 and a temperature of 22 °C or at approximately 10 weeks of storage at a temperature of 4 °C. When stored at a pH of 2 liposomes disintegrated completely into discs at less than 1 day of storage at a temperature of 22 °C or at 5 days of storage at a temperature of 4 °C. Hydrolysis rate constants reported in Table 1 were used to calculate the degree of hydrolysis at these time points. Liposomes stored at a pH of 4 and temperatures of 22 or 4 °C disintegrated completely when DPPC concentrations had been decreased by 5% or 9.9%, respectively. For liposomes stored at a pH of 2, the corresponding reductions in DPPC concentrations were 4.2% or 3.6%.

Our results therefore show that DPPC liposomes containing 4 mol% PEG-lipid in all cases disintegrated completely into discs when DPPC concentrations were decreased by less than 10% and the onset of disc formation occurred at even lower degrees of DPPC hydrolysis. In previous studies of T_C cycled pure DPPC liposomes, the onset of disc formation did not occur until DPPC concentrations had been reduced by approximately 10% as a result of acid-catalyzed hydrolysis [7,8,11]. It thus appears that the presence of PEG-lipids in the membrane considerably increases the disc formation propensity in partially hydrolyzed liposome samples.

4.2. Mechanism of disc formation

We argued previously that disc formation in DPPC/MSPC/DSPE-PEG liposomes occurs due to the presence of lysolipids and PEG-lipids in grain boundaries of the gel-phase membrane [17]. Upon heating, grain boundaries melt at temperatures slightly lower than the T_C of the bulk of the membrane [26,27] and the presence of lysolipid and PEG-lipid promotes dissociation of disc-shaped membrane fragments. Both lysolipids and PEG-lipids are micelle-forming compounds and thus strive to aggregate into structures with a high positive surface curvature. Since the hemispherical rim of a membrane discs presents such a highly curved structure it is favorable for micelle-forming membrane components to remain segregated rather than remix with the bulk phospholipid also in the liquid-crystalline phase. The rim of a bilayer disc is then stabilized in

the presence of lysolipids and, in particular, PEG-lipids and fusion and potential re-closure of large discs into liposomes is thus prevented.

Previously, MPPC and DPPC have been shown to mix homogeneously in large bilayers with negligible surface curvature [28]. We argue, however, that at temperatures lower than the T_C of the formulation the membrane of a 100-nm liposome is different from that of a giant (10 μm) liposome or a flat gel phase bilayer. Bending stresses in 100 nm liposomes lead to membrane inhomogeneities that are noticeable in the polygonal structure of gel-phase liposomes. Although direct evidence is still lacking, it appears likely that micelle-forming membrane components would segregate into the highly curved and relatively disordered membrane areas between flat membrane domains with a higher degree of order.

The observation that DPPC/MPPC/DSPE-PEG_{2000} liposomes (molar ratio: 78:18:4) remained intact at a temperature of 48 °C but disintegrated when the temperature was lowered to 25 °C (Figs. 7 and 8) supports the above assumption. In this formulation, lysolipid and PEG-lipid concentrations were apparently not high enough to promote the formation of membrane discs or other non-liposomal structures in the liquid-crystalline phase. However, since discs formed when liposomes were cooled to temperatures below their T_C, it appears that the phase transition caused an accumulation of lysolipids and PEG-lipids in distinct membrane areas. As an alternative explanation, the cohesive strength of the liposome membrane could have been reduced to such an extent that the stress imposed by the transition from a spherical to a polygonal morphology could not be sustained. Irrelevant of the mechanism, results presented in Fig. 8A and B indicate that liposomes remain intact at temperatures below the T_C of the formulation if grain boundary concentrations of micelle-forming membrane components are below those necessary to trigger disc formation upon cooling. Upon heating to temperatures above their T_C, such liposomes may form pore-like defects at areas where high concentrations of micelle-forming membrane components exist. Further examples for such formulations are DPPC/MSPC/DSPE-PEG_{2000} (molar ratio: 90:10:4) [17], and DPPC/MPPC/DSPE-PEG_{2000} (molar ratio: 90:10:4) [29]. Repeated T_C cycling may, however, induce disc formation even in those liposome formulations [17].

4.3. Structure of hydrolyzed liposomes before phase transition

Since phospholipid hydrolysis increases the disc formation propensity of liposomes their performance may be affected when stored in a basic or an acidic environment. The latter is a common condition when liposomes are prepared with a pH gradient across the membrane for pH gradient mediated drug loading [30,31]. Since the structural reorganization required for disc formation cannot occur until grain boundaries melt, liposomes stay intact at temperatures below the T_C of the formulation even if considerable quantities of lysolipids have accumulated. As shown herein, liposomes stayed intact for more than 12 weeks when stored at a temperature of 22 °C and a pH of 4 although the DPPC concentration decreased by more than 40%. Because such lysolipid-enriched liposomes remain stable at temperatures below their T_C these compositional changes may remain unnoticed. Partially hydrolyzed liposomes will, however, disintegrate readily upon heating to temperatures in the vicinity of the formulation's T_C (Figs. 2 and 4–6). As mentioned in Results, the apparent mean particle size of liposome samples before T_C cycling eventually increased likely as a result of fusion when liposomes were stored for 3–4 weeks at a temperature of 22 °C and a pH of 2 and DPPC concentrations had decreased by approximately 70% or more (Fig. 5D).

4.4. Effects of palmitic acid on the aggregate structure

Partially hydrolyzed liposomes contain not only lysolipids but also fatty acids as the second hydrolysis product. Since apparent pK_a values of fatty acids at physiological salt concentrations range in liposomes from 7.2 to 8 [32] fatty acid molecules were in the present study predominately protonated under the experimental conditions. These molecules then have a propensity to form inverted structures with a net negative surface curvature [33,34]. Nevertheless, the presence of fatty acids appeared to promote, rather than inhibit the disc formation process. Although MPPC membrane concentrations of 9 mol% are not sufficient to cause complete disintegration of DPPC/DSPE-PEG_{2000} liposomes into discs after T_C cycling (Fig. 8A), complete discs transformation occurred when DPPC concentration had been reduced by hydrolysis by 10% or less (Figs. 2 and 4–6). At this time point, lysolipid membrane concentrations were lower than 10 mol% in some cases as low as 3.6% (Fig. 1B) indicating that the presence of fatty acid had increased the propensity of disc formation to occur. A probable explanation for this effect is that membrane additives, such as fatty acid, in grain boundaries weakens their cohesive strength.

Initially, almost equal concentrations of MPPC and palmitic acid are generated by hydrolysis, but as lysolipid membrane concentrations increase, MPPC itself is increasingly hydrolyzed into palmitic acid and the water-soluble glycerol-3-phosphatidylcholine. Consequently, MPPC concentrations reach a plateau and eventually decrease at later time points (Fig. 1B). The decrease in the MPPC/palmitic acid ratio affects both size and morphology of aggregate structures formed after cycling liposomes through their T_C. As this ratio decreases, the apparent mean particle size increases (Fig. 2) and large discs and eventually big bilayer sheets appear in the sample (Fig. 5C). It appears that small discs fuse into larger aggregates when lysolipid concentrations become too low to stabilize the relatively large rim area of a large number of small discs. This process is presumably facilitated by hydrolysis of PEG-lipids, which otherwise would prevent membrane interactions leading to fusion. After extensive hydrolysis MPPC/palmitic acid ratios in the membrane become low enough to allow the formation of more complex structures (Fig. 5D). Structures similar to those in Fig. 5D have previously been observed and identified as intermediates formed during the transition from the lamellar to inverted liquid-crystalline phases [35,36].

4.5. Concluding remarks

The results of the present study stress that storage conditions and the thermal history have to be considered carefully when evaluating properties of liposome preparations. Our results point out that the tendency of liposomes to form alternative aggregate structures is highly dependent on the membrane composition and augmented in the presence of PEG-lipids. A metastable liposome formulation with a high propensity to disintegrate or to form pore-like defects may generally be considered unfavorable for drug delivery applications. This feature can, however, be desirable for certain applications. Drug release from such liposomes can be instantaneous at temperatures close to the T_C of the formulation and this effect has been exploited for tumor-targeted drug delivery using thermosensitive liposomes containing PEG-lipids and MSPC or MPPC as membrane components [37].

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