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Differential scanning calorimetric analysis of dipalmitoylphosphatidylcholine-liposomes upon hydrolysis

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Abstract

Hydrolysis of liposomal dipalmitoylphosphatidylcholine (DPPC) at pH 4.0 or 10.5 and at 30°C resulted in the formation of free palmitic acid (PA), 1- or 2-palmitoyl-*sn*-glycerol-phosphoryl choline (LPC) and glycerophospho compounds. As determined by differential scanning calorimetry (DSC), the presence of even low percentages of PA and LPC had a dramatic, pH-dependent effect on the melting characteristics of DPPC. The pre-transition disappeared at low degrees of hydrolysis. Upon further hydrolysis, the DPPC-peak broadened and decreased in enthalpy and a second peak came up at its left hand side (at pH 10.5) or at its right hand side (at pH 4.0). This phenomenon is related to a difference in ionisation state of PA (at pH 4.0 non-charged and at pH 10.5 charged).

Keywords: Liposomes; Dipalmitoylphosphatidylcholine (DPPC); Chemical and physical stability; Hydrolysis; Differential scanning calorimetry (DSC)

1. Introduction

Differential scanning calorimetry (DSC) has been widely used in the last three decades to assess characteristics of liposome dispersions. Both properties of liposomal phospholipids and interactions of phospholipids with other compounds (drugs, cholesterol, probes, proteins, etc.) have been studied successfully with DSC (Jain and Wu, 1977; Silvius, 1982; Biltonen and Lichtenberg, 1993). In this study we will use the potential of DSC analysis to monitor dramatic effects of chemical hydrolysis of phospholipids on liposomal bilayers with a so-called gel-to-liquid phase transition (such as those used for hyperthermia (see Maryama et al., 1993)).

Liposomes have potential benefits as carriers of drugs, antigens or diagnostic agents (Crommelin and Schreier, 1994). Upon storage, hydrolysis of liposomal phospholipids will results in the formation of free fatty acids, lysophospholipids and glycerophospho compounds (Grit and Crommelin, 1993; Grit et al., 1993). Both fatty acids and lysophospholipids will accumulate in the lipid bilayer upon hydrolysis of liposomal phospho-

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lipids. In contrast, glycerophospho compounds are highly water-soluble and will have no or only a minor effect on the lipid bilayer. This degradation process may affect liposome characteristics 'on the shelf' and in vivo. Other techniques already showed that hydrolysis can change the properties of liposome dispersions. It can lead to a change in bilayer permeability (Grit and Crommelin, 1992), a change in phospholipid conformation from a bilayer structure into a micellar structure (Zuidam, 1994; Zuidam et al., 1995) and, as determined by fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene, in an increase in main phase transition temperature at acidic environment (Zuidam, 1994; Zuidam et al., 1995).

To our knowledge, no calorimetric study on the effect of hydrolysis on the bilayer transitions has been performed before in a systematic way. The only, incomplete, information about the calorimetric effects of hydrolysis on liposomes was provided after autoclaving (15 min at 121°C) of 0.2 μm dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol (DPPC/DPPG) 10/1-liposomes at pH 4.0 (about 3-7% increase in hydrolysis) and at pH 7.4 (about 1% increase in hydrolysis) (Zuidam et al., 1993). Upon autoclaving at pH 4.0 the pre-transition peak disappeared and a shoulder appeared at the right-hand side of the main phase transition. At pH 7.4 the differences before and after autoclaving were less pronounced: the melting enthalpy (ΔH) of the pre-transition tended to decrease and a small shoulder was monitored at the left-hand side of the main peak. The difference in the position of the shoulders is probably related to the non-charged state of fatty acids in bilayers at pH 4.0 and a partially charged state at pH 7.4 as the apparent pK_a of palmitic acid in DPPC-bilayers has been reported to be about 7.5 (Férnandez et al., 1986; Cevc et al., 1988).

In this study, non-sized DPPC-liposomes were hydrolysed at pH 4.0 (all fatty acids non-charged) or pH 10.5 (all fatty acids charged) at 30°C. This composition of liposomes, only DPPC, was chosen, because it is one of the most widely studied phospholipids, and calorimetric studies have been performed before to assess the individual effects of the two degradation products palmitic acid (PA) (Mabrey and Sturtevant, 1977; Schullery et al., 1981) and 1- or 2-palmitoyl-*sn*-glycerol-phosphorylcholine (LPC) (Van Echteld et al., 1980) on the phase transitions of DPPC-liposomes. Turbidity measurements were performed in parallel to gain additional information about the phospholipid morphology of the dispersions.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC > 99% pure) was a gift from Nattermann Phospholipid (Cologne, Germany). Monopalmitoylphosphatidylcholine (LPC > 99% pure) was obtained from Avanti Polar Lipids (Pelham, AL, USA). All other chemicals were of analytical grade. The water was double-distilled before use.

2.2. Preparation of liposome dispersions

Liposomes were prepared by the 'film' method. An appropriate amount of DPPC was 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 50 mM acetate buffer (pH 4.0) and 0.12 M NaCl or 50 mM glycine buffer (pH 10.5) and 0.12 M NaCl. At 30°C the pH of the dispersion was measured and adjusted, if necessary. The liposome dispersions were refrigerated overnight. Subsequently, the pH of the dispersion was measured again at 30°C and adjusted, if necessary. The final phospholipid concentration was about 200 mM.

2.3. Hydrolysis experiments

The prepared liposome dispersions were filled into ampoules under a nitrogen atmosphere and sealed. Ampoules were stored in a constant temperature water bath at 30°C. Samples were taken after appropriate time intervals.

DSC and turbidity measurements were done immediately and then the liposomes were stored in the freezer (− 20°C) until chemical analysis. Experiments were performed in triplicate.

2.4. Differential scanning calorimetry

Seventeen μl of liposome dispersion were put into an aluminium pan. As a reference, an empty aluminium pan was used. Both pans were sealed so that water could not evaporate during measurements. Calorimetric scans from 30 to 60°C were performed on a Netzsch DSC 200 low-temperature DSC (Netzsch Gerätebau, Selb, Germany). This is a temperature controlled heat-flux DSC system. The scanning rate was 2°C/min. Each sample was measured at least twice.

2.5. Turbidity measurements

Turbidity of 30 times diluted liposome dispersions was measured at 450 nm in a 1-mm cuvette on a Pye Unicam Pu 6800 UV/VIS spectrophotometer (Philips/Pye Unicam, Cambridge, England) at ambient temperature. Turbidity depends on several parameters such as concentration, size and batch (Barenholz and Amselem, 1993). To compare the different batches, only the relative turbidity (compared to the turbidity of fresh liposomes of the same batch) is shown in this study.

2.6. Analytical methods

Phospholipids were analysed by HPLC as described earlier (Grit et al., 1991). Samples for the HPLC analysis were prepared by the Bligh and Dyer extraction method (Bligh and Dyer, 1959). The phospholipids were collected in the chloroform phase. After dilution of the chloroform phase in methanol, aliquots of 100 μl 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). Peak areas were measured with a computer-controlled integrator-based data system (WOW, Thermo Separation Products, Fremont, CA, USA). The separation of the phospholipids was carried out on a Zorbax aminophase column (25 cm × 4.6 mm, i.d., 5-μm particle size, Du Pont Company, Wilmington, DE, USA) at 35°C. An Adsorbosphere NH_2 5μ-guard column (Alltech Associates, Deerfield, IL, USA) was connected before the Zorbax aminophase column. The mobile phase consisted of a mixture of acetonitrile/methanol/5 mM ammonium dihydrogen phosphate solution, pH 4.8 (62/28/6, v/v). The flow rate was 1.5 ml/min.

Glycerophospho compounds (GPC), the (actual) end products of hydrolysis of phospholipids, were measured by phosphate determination according to Fiske and Subbarow (Fiske and Subbarow, 1925) in the supernatant of a Bligh and Dyer extraction mixture (Bligh and Dyer, 1959).

3. Results

DPPC-liposomes were hydrolysed at pH 4.0 or 10.5 and at 30°C. The concentrations of DPPC and its degradation products were measured and are shown as the percentages of total phosphate concentration as function of time and as function of % hydrolysis in Fig. 1. As before (Zuidam et al., 1993), the % hydrolysis of the liposome dispersions in this study was defined as

$$\%\ hydrolysis = 100\% - [DPPC] \times 100\%/([DPPC] + [LPC] + [GPC]) \quad (1)$$

where [DPPC] is the concentration of DPPC, [LPC] is the sum of the concentrations of 1- and 2-palmitoylphosphatidylcholine (the first hydrolysis product of DPPC) and [GPC] is the concentration of glycero phosphatidylcholine (the (actual) end hydrolysis products of DPPC). The relative concentration of palmitic acid (PA) was calculated from the increase in LPC and GPC (equal to formation of 1 and 2 units PA, respectively). The disappearance of DPPC in buffered dispersions followed pseudo first-order kinetics as was indicated by straight lines upon plotting of the semilogarithmic values of the relative concentration of DPPC against time (results not shown). The pseudo first-order rate constants (k_{obs}) derived from these straight lines were 1.1×10^{-7}

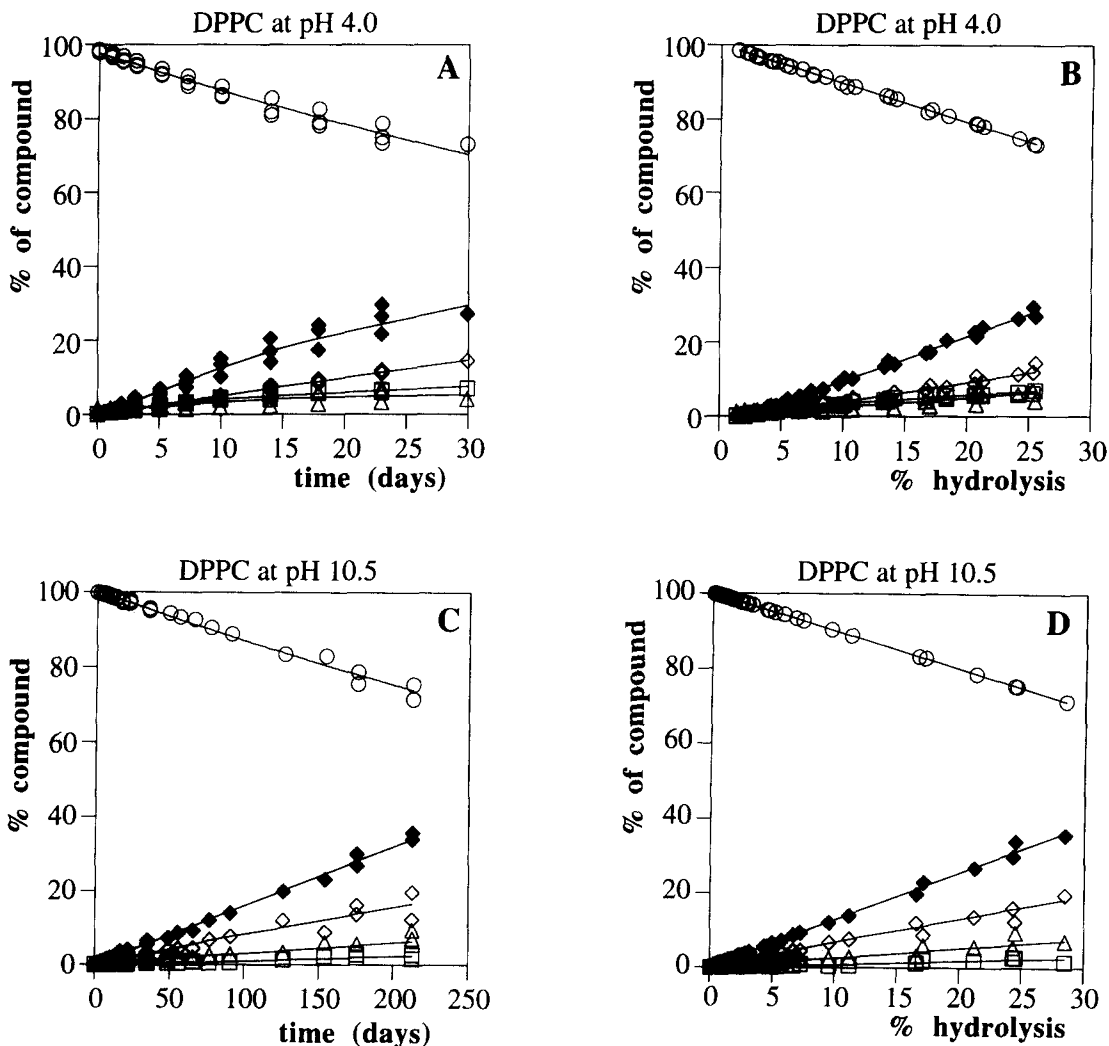

Fig. 1. Composition of liposome dispersions upon hydrolysis of liposomal DPPC at pH 4.0 or 10.5 and 30°C against time (A or C, respectively) or % hydrolysis (B and D, respectively). ○, DPPC; □, 2-palmitoyl-*sn*-glycerol-phosphorylcholine; ◇, 1-palmitoyl-*sn*-glycerol-phosphorylcholine; △, GPC; ◆ PA.

(S.D. 0.1 × 10^{-7} s^{-1}) or 1.7 × 10^{-8} (S.D. 0.1 × 10^{-8} s^{-1}) at pH 4.0 or 10.5, respectively.

In Fig. 2A and B the results of turbidity measurements of DPPC-liposomes at ambient temperature are shown before and after heating for 1 min at 70°C as a function of the % hydrolysis. Before heating at 70°C, hardly any change was observed upon hydrolysis of DPPC-liposomes at pH 4.0 or 10.5 and 30°C. However, after heating at 70°C major changes were observed. Above a critical hydrolysis percentage of about 10%, the turbidity of DPPC-liposomes at pH 4.0 and 10.5 decreased. Upon further hydrolysis, the turbidity increased, probably due to formation of aggregates with high fatty acid content (as could be clearly visually observed upon prolonged storage; results not shown).

In Fig. 3A–D typical DSC scans of fresh and hydrolysed DPPC-liposomes are shown. Upon hydrolysis of DPPC-liposomes at pH 4.0 the gel-to-liquid phase transition broadened while the pre-transitions disappeared above 7% hydrolysis.

Further hydrolysis resulted in a decrease of the DPPC-peak at about 42°C and the appearance of a second peak at higher temperatures. The melting enthalpy (ΔH) of the DPPC-peak decreased while the total ΔH remained constant after an initial increase of about 20–30% upon hydrolysis. The onset temperature (T_{on}) of the DPPC-peak only showed a minor decrease upon hydrolysis (from about 41.6 to 41.0°C). The DSC scans changed upon repeating the runs once. The scans did not or hardly change upon further repeating of DSC measurements.

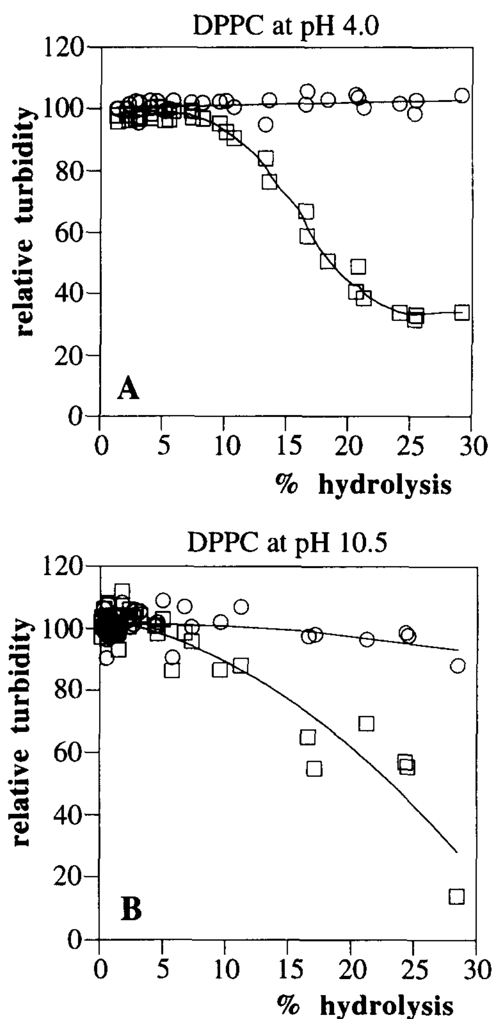

Fig. 2. Relative turbidity at 450 nm against % hydrolysis of DPPC-liposomes at pH 4.0 (A) or 10.5 (B) before (○) and after (□) heating for 1 min at 70°C.

Upon hydrolysis of DPPC-liposomes at pH 10.5, very different effects were observed than upon hydrolysis at pH 4.0. Also here, the gel-to-liquid phase transition broadened and the pre-transitions disappeared (above 3%). However, now a second peak came up at the other, left hand, side of the main peak upon hydrolysis. This second peak was already observed at lower hydrolysis percentages than the second peak upon hydrolysis of DPPC at pH 4.0 (at $\geq$ 2% hydrolysis). Especially, in the second heating scans these second peaks were clearly observed. The presence of the second peak resulted in a gradual shift of T_{on} from about 41.6 to 39.4°C. The scans did slightly change upon further repeating of DSC measurements. The total ΔH of the liposomes hardly changed upon hydrolysis at pH 10.5; a gradual increase of about 10% was found after 20% hydrolysis.

4. Discussion and conclusions

DPPC-liposomes were hydrolysed in this study at pH 4.0 or 10.5 and at 30°C. Surprisingly, the k_{obs} of DPPC at pH 10.5 was about 6-fold lower than the k_{obs} at pH 4.0. This is not expected from studies performed by Grit et al. and other groups (Grit and Crommelin, 1993, and references in this review). In these studies liposomes were hydrolysed in the pH range 3–9. The hydrolysis of liposomal phospholipids is catalysed by protons and hydroxyl ions and reaches a minimum at about pH 6.5. Upon plotting the logarithmic values of k_{obs} against the pH, a V-shaped pH profile for the hydrolysis process was obtained. Extrapolation of the basic branches of several V-shaped pH profiles indicated that the value for k_{obs} of liposomal phospholipids at pH 10.5 should be equal to or larger than the k_{obs} at pH 4.0 instead of lower as observed. At the moment, we cannot explain this discrepancy. One difference between the experimental conditions used by Grit et al. and the present study is the buffer system. They never used glycine buffers.

The individual effect of the two degradation products PA and LPC on the phase transitions of DPPC-liposomes have been published before. In-

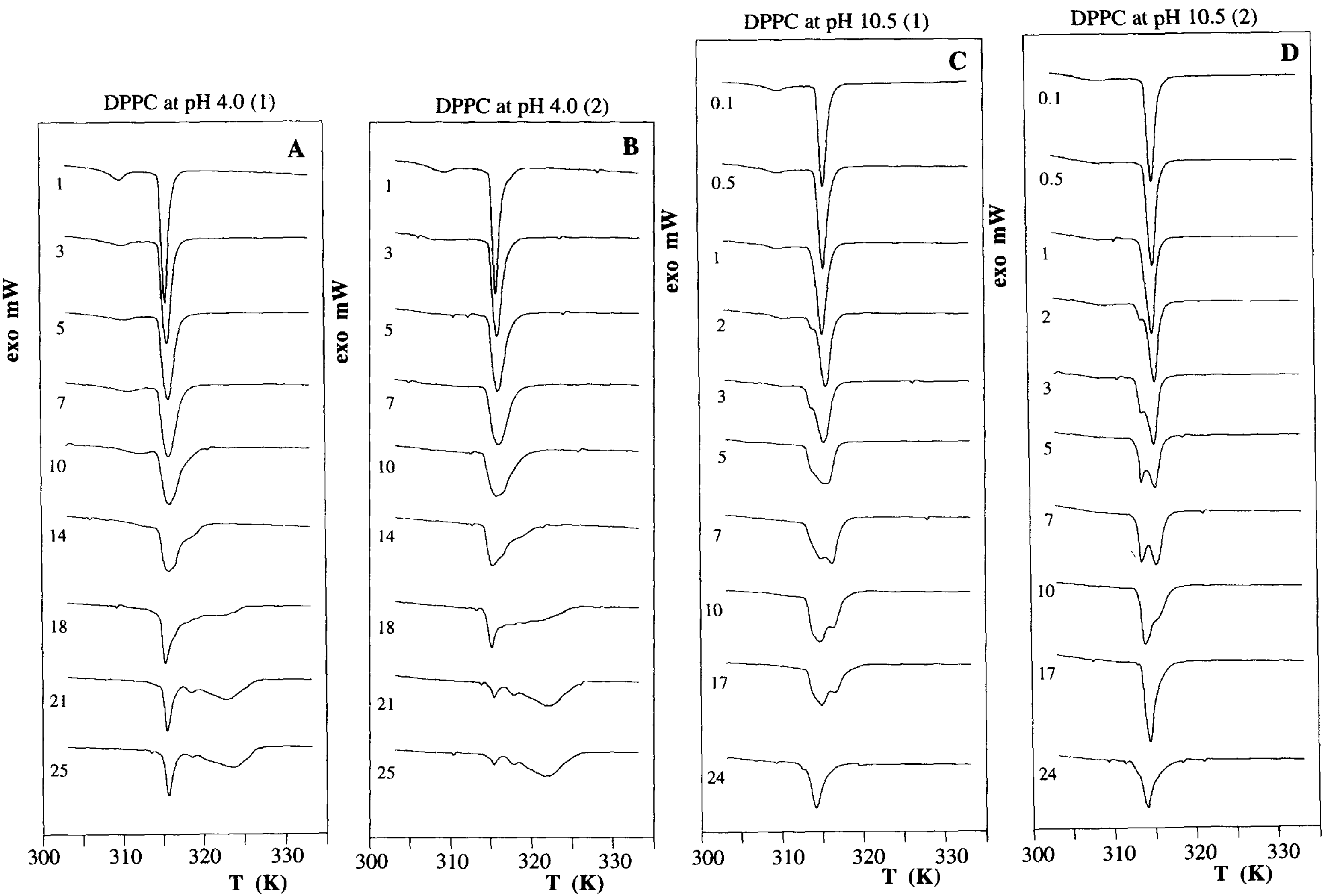

Fig. 3. Typical DSC scans of non- and hydrolysed DPPC-liposomes at pH 4.0 (A and B) or 10.5 (C and D) and at 30°C. A and C are the scans obtained in the first heating cycle and B and D the scans obtained in the second heating cycle. The numbers in the figures at the left indicate the % hydrolysis.

corporation of up to 40 mol% of LPC in DPPC-liposomes at pH 7.0 resulted in only one sharp transition, while the pre-transition had disappeared at 10 mol% (Van Echteld et al., 1980). The sum of ΔH of the pre-transition and the main transition showed a linear decrease only after incorporation of over 20 mol% LPC. The addition of only PA also resulted first in disappearance of the pre-transition (above about 6 mol%), while subsequently the main phase transition broadened and shifted to higher temperatures (Mabrey and Sturtevant, 1977; Schullery et al., 1981). Very different effects were seen upon addition of sodium palmitate (PA in an ionised form) to DPPC-bilayers: the pre-transitions remained up to 36 mol% palmitate and the main phase transition hardly broadened and shifted only a few degrees to lower temperature (Schullery et al., 1981). In the present study, we show for the first time the effects of PA and LPC (together) on melting properties of DPPC-bilayers; their presence is the result of chemical hydrolysis of DPPC. The observed changes in the DSC scans are comparable with changes obtained by addition of palmitic acid (when hydrolysed at pH 4.0) or sodium palmitate (when hydrolysed at pH 10.5). This is in agreement with the observation described above that incorporation of high concentrations of LPC (up to 80%) in DPPC-liposomes at pH 7.0 only resulted in minor broadening of the main peak (Van Echteld et al., 1980). However, when PA was in an ionised form in this study (at pH 10.5) only a shoulder was observed at lower temperatures upon hydrolysis of DPPC (see Fig. 3), while the DPPC-peak did not shift to lower temperatures (as shown in the study by Schullery et al., 1981). This discrepancy is probably due to the presence of LPC in the DPPC-bilayers in this study.

Thus, the appearance of the second peak at different sides of the main transition peak is dependent on the ionisation state of PA. As monitored by the growth of a second peak in the DSC scans, hydrolysis resulted in the presence of several domains differing in palmitic acid content within one lipid bilayer. Thus, PA caused a more pronounced effect on the melting transitions of DPPC-bilayers than LPC.

At high hydrolysis levels, the DSC scans of liposomes changed upon repeating the runs (see Fig. 3). The two different peaks observed in those scans tended to revert to one peak. Probably mixing of the bilayer compounds occurred. Another possibility is that the changes in the repeated DSC scans reflect hysteresis. Such a hysteresis phenomenon has been described before by us upon measuring fluorescence anisotropy values of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in hydrolysed liposomal bilayers composed of 0.2 μm DPPC/DPPG 10/1 at pH 4.0 (Zuidam, 1994; Zuidam et al., 1995). Above the critical hydrolysis percentage, anisotropy values measured during a cooling cycle were not identical to the anisotropy values measured during a heating cycle; those measured during cooling shifted to lower temperatures compared to the anisotropy values measured during heating. Such a phenomenon was found before for stearoylsphingomyelin bilayers (Estep et al., 1980). Probably 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. 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, LPC and PA upon hydrolysis. However, the exact nature of a possible metastable gel structure is not known yet. The various options include interdigitation of lysophospholipids (Slater and Huang, 1988).

Turbidity measurements were performed to gain information on the morphology of the liposomal phospholipids. Upon hydrolysis the turbidity showed a pronounced decrease above a hydrolysis percentage of about 10% at pH 4.0 and 10.5 when the liposome dispersions were heated for 1 min at 70°C. A more gradual drop was observed with the pH 10.5 dispersion. This drop

in turbidity upon heating was found previously (Zuidam, 1994; Zuidam et al., 1995). The drop in turbidity monitored the conversion of the liposomes from a bilayer into a micellar system. As a result of hydrolysis, liposomes can undergo such a reorganisation if (1) the liposomes pass through a gel-to-liquid phase transition during a heating or cooling run, and if (2) the chemical hydrolysis level exceeds a critical hydrolysis percentage (or the phospholipid bilayer must contain critical amounts of LPC and PA). This reported critical hydrolysis percentage was demonstrated to depend both on the chain length and on the head group of the liposomal phospholipids. At pH 4.0, the DSC scans changed upon repeating the runs if the liposomes changed from a bilayer into a micellar system as monitored by turbidity measurements. This happened above the critical hydrolysis percentage of about 10%. However, at pH 10.5 the relationship between turbidity drop and the changes in the DSC scans (upon repeating the runs) was much less clear. Thus, at pH 10.5 the occurrence of major differences between repeated DSC scans cannot be fully attributed to the conversion of the physical conformation of the phospholipids as monitored by turbidity changes.

The above-mentioned measurements of the fluorescence anisotropy of the lipophilic probe DPH in 0.2 μm DPPC/DPPG 10/1-liposomes of different hydrolysis levels also demonstrated that bilayer rigidity below and above the T_m did not change upon chemical hydrolysis and upon formation of micelles (Zuidam, 1994; Zuidam et al., 1995). 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 at temperatures below T_m. However, this was not found. Therefore, we hypothesized 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.

The present study support this hypothesis. The observed changes in the DSC scans of hydrolysed DPPC-liposomes are comparable with changes obtained by addition of palmitic acid (when hydrolysed at pH 4.0) or sodium palmitate (when hydrolysed at pH 10.5) to DPPC-bilayers. The total ΔH did not decrease, as might be expected when micelles are formed, but increased 10-30% upon hydrolysis.

In conclusion, DSC analysis is a very powerful technique to monitor pH-dependent physical changes to the DPPC-bilayers as a result of hydrolysis. We hypothesize that disk-shaped micelles were formed upon hydrolysis of DPPC-liposomes. However, other techniques should provide more information about the exact structure of the micelles.

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