Cardiolipin in phospholipid bilayers

A calorimetric study

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Abstract:

The purpose of this study was to understand cardiolipins behavior in different pH: 5.5, 7 and 9. Cardiolipins miscibility in other bilayers as 1,2-dimyrilstoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were also studied. The instrument used in this study was a differential scanning calorimetry. The conclusions that could be drawn from the scans were that cardiolipin has different phase behavior in different pH because of the charge and that the phase behavior get more complex with higher charge. With the mixed systems with cardiolipin and DMPC it was shown that cardiolipin was miscible in DMPC bilayers with a low concentration cardiolipin and that the miscibility decreased with increased concentration of cardiolipin. But with higher pH values cardiolipin was miscible in even higher concentrations of cardiolipin. This indicates that with higher charge on the cardiolipins headgroup it is easier for cardiolipin to dissolve in a bilayer. It was also show that cardiolipin was easier dissolved in bilayers in liquid crystalline phase as DOPC, than bilayers in a solid phase as DMPC.

Populärvetenskaplig sammanfattning

Cellerna i kroppen speciella cellmembran som består till största delen av protein och lipider och kolhydrater. Det finns flera sorts lipider, de består av en huvudgrupp som kan ha olika laddning och som är hydrofil, vilket innebär att den är vatten älskande samt en eller flera kolsvansar som är hydrfoba, det vill säga, ogillar vatten. Dessa lipiderna kommer organisera sig i strukturer av bilager i vattenmiljöer. De vattenogillande kolkedjorna vänder sig då mot mitten och de vatten älskande huvudgrupperna mot vattnet. Dessa bilagerna kan ha olika fas beteende i den meningen att kolkedjorna kan vara i ett fast eller flytande tillstånd beroende på temperatur, pH, salt koncentration etc.

Den lipiden som studeras i denna studie heter cardiolipin. Det är en lipid med fyra kolkedjor och har en huvudgrupp som kan ha en laddning på -1 till -2 beroende på pH. Frågorna som denna studie kretsar runt hur cardiolipins fasbeteende beror på pH, och hur bra cardiolipin löser sig i andra lipidbilager. Detta undersöktes via en kaliometri-metod. En kaliometer mäter hur mycket energi, i form av värme, som krävs för att en lipid ska ändra fas.

Resultatet som kunde ses från denna studie var att cardiolipins fasbeteende såg olika ut i olika pH på grund av de olika laddningarna cardiolipin har vid olika pH. Vid högre laddning på cardilolipin så var det ett mer komplext fasbeteende. När cardiolipins blandbarhet med andra bilager undersöktes så kunde det konstateras att vid små koncentrationer av cardiolipin så lyckades cardiolipin blanda sig i bilagret. Men med ökad koncentration av cariolipin så minskade blandbarheten i bilagret. Vid höga pH blandade sig dock cardiolipin även vid de högre koncentrationerna av cardiolipin. Detta beror antagligen på den högre laddningen på huvudgruppen vilket gör att lipiderna repellerar varandra och kan slinka in i lipid bilagret enklare. det kunde även visas att det var enklare att blanda cardiolipin i ett bilager med flytande kolkedjor än ett bilager med fasta kolkedjor.

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1. Introduction:

Cardiolipin is an important component of the membrane of mitochondria(1). It is also considered important for the interaction between these membranes and other biomolecules. Recently, it has been shown that α-synuclein, which is associated with Parkinson's disease, shows strong affinity for membranes containing cardiolipin (2).

Cardiolipin is different from other common lipids, it has four lipid chains and under certain conditions high charge (-2). The question raised in this project is: How does cardiolipin affect membranes properties? Is it soluble in phosphosfolipid bilayers, and how does the phase behavior depend on pH (cardiolipin charge)? This project is focused on the phase transitions in response to changes of temperature and different mixed systems with cardiolipin and 1,2-dimyrilstoyl-sn-glycero-3-phosphocholine (DMPC) at different pH, with Differential scanning calorimetry (DSC) as method.

2. Background:

2.1 Lipid membrane:

The cell membranes consist of many different kinds of proteins and lipids see fig 1. The three most common of lipids that exists in the cell membranes is phospholipids, sterols and glycolipids(3). The composition of different lipids can vary between different membranes, but the most common lipids are the phospholipids. There are many different phospholipids and the most common is phosphatidylcholine (PC). One example of PC is DMPC, see fig 3, it has a zwitterionic choline headgroup and two 14C hydrocarbon chains. There also exist anionic lipids, for example Phosphatidylserine (PS) which contains two hydrocarbon chains and a negatively charged serine headgroup (-1)(4).

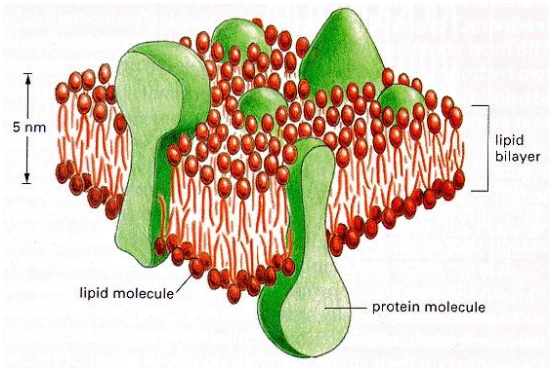

Figure 1: Schematic picture of the cell membrane (15).

Cardiolipin, see fig 2, exist in the mitochondria membrane, this diphospatidylglycerol lipid, which have 4 hydrocarbon chains and can have a negative charge up to -2. It has two pKa values, pKa_1=2,8 and pka_2, often referred to as pKa_2= >7,5 by literature (5).

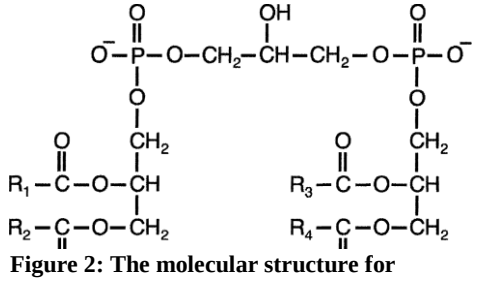

Figure 2: The molecular structure for cardiolipin(18).

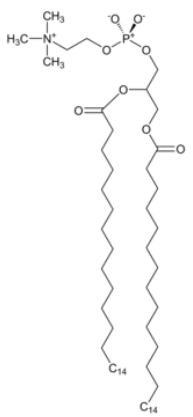

Figure 3: The molecular structure for DMPC(19).

2.2 Amphiphilic molecules:

Amphiphilic molecules are molecules that have one polar/hydrophilic part and one nonpolar/hydrophobic part typically made of hydrocarbon. Lipids are amphiphilic molecules which mean that they consist of a headgroup that is hydrophilic and a tail which is hydrophobic. When lipids are present in a water environment, they will self organize to form structures where the polar headgroups face the water and the hydrophobic tails face each other. This organization is driven by the hydrophobic effect, but the precise structure depends on the interaction between the headgroups and the hydrocarbon chain, and between the lipids and the solvent. These interactions between the lipids depend on e.g. the shape of the lipid chains, the lipid concentration and the solution conditions, for example pH, salt concentration and temperature. (6)

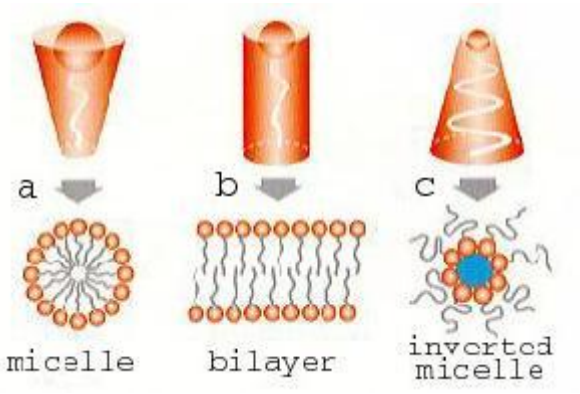

Figure 4: Schematic picture of lipids self organization (16).

For example, can lipids like DMPC have cylindrical shape and form lamellar phases(fig 4, b). Amphiphiles, on the other hand, with a large head group and a small hydrophobic part have a tendency to form micelles(fig 4, a), and amphiphiles with bulky chains and small headgroups preferably form inverted structures(fig 4, c) (6).

2.3 Lamellar phases:

The lamellar phases can form from lipids with either liquid or solid chains(7). When the lamellar phase is in a liquid crystalline phase (L_α), the lipid chains are in a melted state, the chains are disordered and water can diffuse freely between the bilayers of the lamellar phase. The crystalline lamellar phases are solid structures, which may include a few water molecules bound tightly to the lipid headgroups and have highly ordered solid chains. There are different kinds of gel phases, and they can be seen as an intermediate between the liquid crystalline and the solid crystalline phase. The bilayers of the gel phase are solid, just as the solid crystalline

bilayers, but water freely diffuse in the layers between the bilayers in the lamellar phase as in the liquid crystalline phase. There exist different types of gel phases, for example for the lipid DMPC there are two see fig 5, one called the L_β, that have tilted lipid chains and P_β which is a rippled gel phase (8). In many cases a transition can be induced between liquid crystalline lamellar phase to gel and crystalline lamellar phase by, e.g., reducing temperature or water content (8).

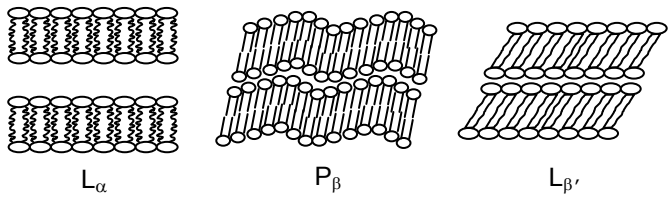

Figure 5: A Schematic drawing of the three lamellar phases. (17)

Lamellar phases can be dispersed to vesicles, which is a lamellar phase in a spherical structure that contains solvent on the inside. There are different kinds of vesicles with multi-lamellar and unilamellar arrangements. The multi-lamellar consist of several bilayers whereas unilamellar only consists of one bilayer. In this study the lamellar phases are through freeze-thawing made into multi-lamellar vesicles. Unilamellar vesicles can be made through extrusion, which squeezes the lamellar phase through small pores, or sonication where ultrasound is used. (9) The vesicles are generally not equilibrium structures and will eventually go back to the lamellar phase (7)

2.4 Phase transitions:

When a phase transition occurs there will be a distinct ~~a~~ change in the aggregate structure in response to a small change in the sample environment. Phase transitions can be induced by changing the temperature, salt concentration, pH etc(10). There are different transitions that can occur, as solid – solid transitions, e.g. between different gel phases, liquid- liquid transition between different liquid crystalline phases, or solid-liquid transitions between, e.g. a gel phase and a liquid crystalline phase. Transitions between phases give rise to an enthalpy effect. The transition between a gel phase and a liquid crystalline phase is generally referred to as the main transition, which generally has a large enthalpy effect. The reason why main transition has a large enthalpy effect is because when the chains melt the van der Waals interaction breaks and this generates more energy than the pre-transition. Pre-transition occurs at gel-gel transition, where there is a little change in the molecular organization and therefore

has a smaller enthalpy effect. The main transition occurs at the chain melting temperature, T_m. The T_m depends on the length of the lipid chain, the number of double bonds in the chain and on the interaction between the headgroups and between the lipid and the surrounding solution (7). For lipids with longer chains, T_m is higher because longer chain offers larger van der Waals forces than short chains. When the lipid chains consist of double bonds the chain melting temperature decrease because the lipids are not as well packed. (7)

2.5 DSC:

DSC is a method that can be used to detect phase behavior and enthalpy effects in a given sample(11). The instrument contains two cells, one measuring cell and one reference cell,

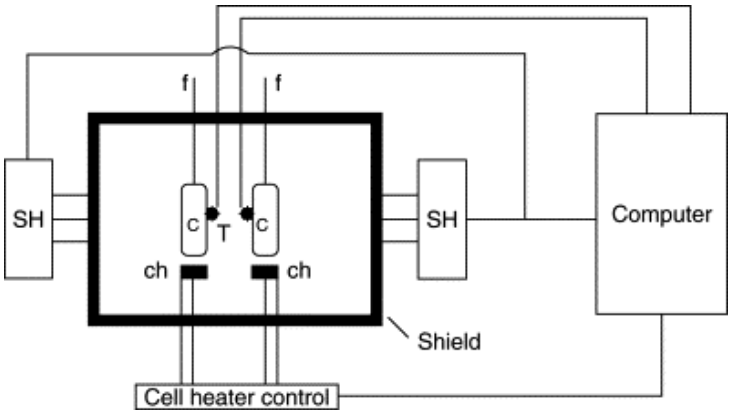

Figure 6: Schematic drawing of a vp DSC(11).

see figure 6. What is measured is the required input of energy to keep the same temperature in the sample and reference cell while the temperature is increased in both cells. This energy is then measured as a function of the temperature and will indicate when a phase transition occurs. The process can be endothermic or exothermic. For the endothermic processes, higher energy input is needed to compensate for the temperature change between the two cells. The DSC reference cell is generally filled with the same solution that the sample is diluted in, and both the sample cell and reference cell are in an adiabatic chamber that is shielded from heat exchange from the surroundings. The DSC microcalorimeter used here is a sensitive instrument that can register small energy changes. The difference between the cells when it comes to the input of thermal power will show up as a peak in the recorded thermogram, when integrating the peak area the enthalpy for the phase transition is obtained (expressed in J/mol if the concentration is known)(11). For example see figure 7, the thermogram is obtained from a DSC scan of DPMC. As seen in the thermogram there are two transitions, the transition that occurs at the lowest temperature is the pre- transition (L_β to P_β) and compared with the main transition (P_β to $L\alpha$) that occurs at T_m = 24°C, it has a significantly smaller enthalpy effect.

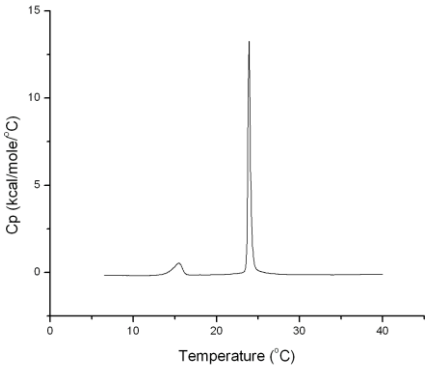

Figure 7: DMPC in water

3. Methods:

3.1 Preparation:

A stock solution of DMPC and Cardiolipin (C14:0) was dissolved in the mixture of chloroform/methanol (2:1), with a concentration of 10 mg/ml. DMPC/cardiolipin was prepared in the following molecular ratios 100:0, 95:5, 85:15, 70:30, 0:100. To each preparation the right amount of lipid mixture with a concentration of 0,6mM was mixed and dried under a stream of nitrogen. The dried film of lipid was left over night under vacuum at room temperature to get rid of the last trace of solvent. After this, three different buffers were used, Mes buffer pH 5,5, tris-HCl buffer pH 7, tris-HCl buffer pH 9 with buffer strength 10 mM and ion strength 10 mM , to hydrate the lipids and enable them to aggregate in a lamellar phase, an excess solution was used. The solution was heated in a warm water bath above the chain melting temperature. After this the sample was freeze-thawed in ethanol with dry ice, and then warmed in a water-bath at temperature above chain melting temperature, this procedure was done approximately five times. The preparations were stored in freezer (-18° C) when not in use. This preparation way was used for all mixtures except for the 100% pure cardiolipin mixture. It was stored in a freezer with a lower temperature (-25C°) for 24 h, to ensure equilibration of cardiolipin solid phases. All the samples were replicated twice.

3.2 DSC:

DSC measurements where preformed at a VP DSC (MicroCal Inc. North Hampton USA). The samples was first degassed and then the reference cell was filled with ca 0,5 ml buffer and the sample cell with ca 0,5 ml lipid solution with the corresponding buffer. The scans were run from 5 to 60 C° with a scan rate of 60 °C/h. At the different measurements, different numbers of scans were used. The scan schedule used for the first measurements was four scans, then termostating at 60° C for 30 minutes and then another four scans from 5 to 60°C. As the incubation at higher temperature did not affect the measured DSC traces, the scanning schedule was later on changed to consist of only 4 -5 scans and no termostating at 60°C. Between the measurements the DSC was cleaned with 10% decon and the buffer changed in the reference cell. The thermograms was processed and analyzed in the software Origin.

4. Results:

In this project the binary system DMPC-water and cardiolipin-water have been studied to understand the phase behavior of cardiolipin in different pH. Three different buffers with pH 5,5; 7 and 9, are of interest because cardiolipin has according to literature two pka values, pka_1= 2,8 and pka_2= 7,5-9,5 (5). This means that at pH 9, cardiolipin is expected to have charge of -2 and at pH 5.5, it is likely to have a charge of -1. At pH 7 charge is less well-defined for cardiolipin. A study on ternary system DMPC-cardiolipin-water for the three different pH's was also made to understand if cardiolipin was able to dissolve in another lipid bilayer.

4.1 Binary lipid-water systems:

4.1.1 DMPC-water system:

The binary DMPC- water system was studied at pH 7 (in pure water)(fig 2.1) and at pH 5,5 (fig 2.2). Characteristics for DMPC-water system is the two phase transitions. The first one is the pre-transition. This transition is between the L_β and P_β phase and for DMPC in pH 5,5 it occurred at T_{pr} = 14°C. The next transition is the main transition and where the chain melting occurs, the transition is between P_β and L_α phases and for DMPC in pH 5,5 it happened around 24° C. DMPC in water gave rise to nearly the same T_m as DMPC in Mes buffer pH 5,5 (see table 1 and fig 2.2). And both DMPC in pH 7 and 5,5 had values around the litterateur value (see table 1).

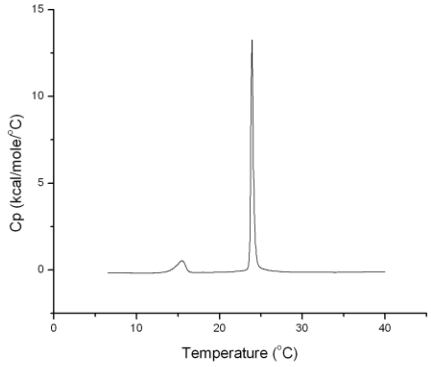

Figure 2.1: DMPC in water

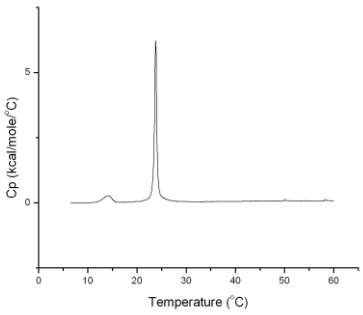

Figure 2.2: DMPC in pH 5,5

Table 1: temperatures and enthalpies for pre-transition and main transition for DMPC

DMPC in	Pre- transtion		Main-transtion	
	T_{pr} (°C)	ΔH_{pr} (kcal/mole)	T_m (°C)	ΔH_m (kcal/mole)
pH 5,5	14	0,24	24	4,7
water	15	*	24	*
litt	14	1,1	24	5,9

*Lipid concentration not well-defined, therefore only transition temperatures are listed.

4.1.2 cardiolipin-water systems :

pH 5,5:

As seen in fig 2.3 pure cardiolipin in Mes pH 5,5 buffer shows only one transition with a sloping baseline, the transition is the chain melting transition that have a T_m around 44°C and an enthalpy effect of 9900 cal/mole.

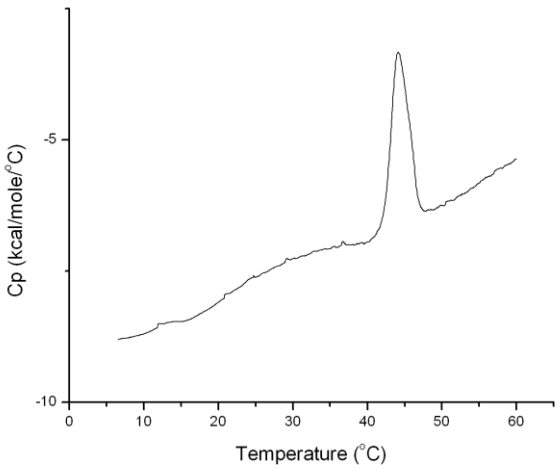

Figure 2.3: pure cardiolipin in pH 5,5

pH 9:

For cardiolipin in tris HCl buffer pH 9 there are three phase transitions detected, see fig 2.4. The first transition is around 15° C and has a enthalpy effect of 1800 cal/mole. The second transition is slightly smaller and occurs around 24°C and has an enthalpy effect of 1200 cal/mole. The main transition occurs around 37°C and has a shoulder, the enthalpy for this peak is 13000 cal/mole. This result corresponds to the previous findings in the literature for cardiolipin in a buffer of high ion strength and pH 7,4 (12).

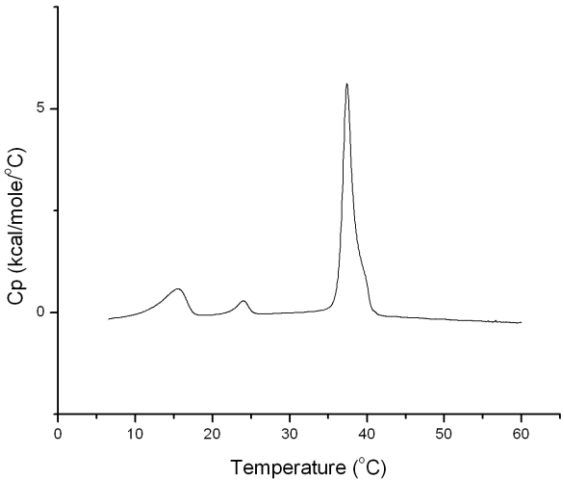

Figure 2.4: pure cardiolipin in pH 9

pH 7:

Cardiolipin in tris HCL pH 7 buffer have four transitions, see fig 2.5. The first transition is around 15°C and has an enthalpy effect of 1200 cal/mole. The second transition is around 26°C and has a higher enthalpy effect of 3700 cal/mole. The main transition can be seen at the two maximum temperatures T1= 37 and T2= 44, and has a total enthalpy effect of 9000 cal/mole. The two main transition temperatures are similar to the T_m for cardiolipin in pH 9 and 5,5, which implies that cardiolipin in pH7 have components that can be recognized from both pH 9 and pH 5,5. However, the measured enthalpies are not comparable between buffers. It is noted that as we don't know how much of each component that we have (-1 and -2 cardiolipin) and it is difficult to make quantitative comparisons of the enthalpy effects. This will be further discussed in section 6.

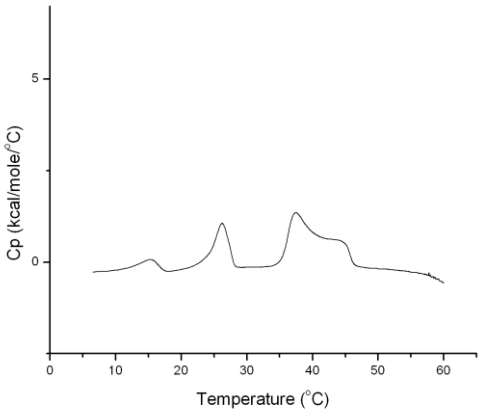

Figure 2.5: pure cardiolipin in pH 7

Table 2: The temperatures and enthalpies for pure cardiolipin in different pH.

Cardiolipin in	Transitons	
	T (°C)	ΔH (cal/ mole)
pH 5,5	44	9900
pH 9	15	1800
	24	1200
	37	13000
pH 7	15	1200
	26	3700
	37 and 44	9000

4.2 Ternary DMPC-cardiolipin-water systems:

In the following text a study on ternary system DMPC-cardiolipin-water have been made to understand if cardiolipin is able to dissolve in a another phospholipid, DMPC, and study the effect of different pH.

4.2.1 DMPC-cardiolipin mixtures at PH 5,5:

Figure 3.1 shows DMPC/Cardiolipin 95:5, in the thermogram a pre-transition at T_{pr} = 18°C can be seen with an enthalpy effect of 360 cal/mole, and a main transition which occur at T_m= 25°C with a higher enthalpy effect of 4900 cal/mole. The DSC trace obtained for this mixture is rather similar to that obtained for DMPC. The pre-transition is observed also in the presence of cardiolipin, but it is shifted to a slightly higher temperature. This is also the case for the main transition. Another major difference is that the transition peaks are significantly broader, ΔT= 8°C, in the ternary mixture, indicating the presence of a 2-phase gel-liquid crystalline co-existence region.

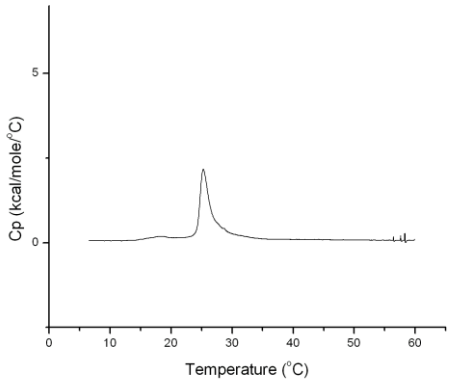

Figure 3.1: 5 mole% cardiolipin in DMPC in pH 5,5

Increased cardiolipin concentration resulted in a more complex phase behavior as can be seen from DSC traces in Fig 3.2 and 3.3. In Fig 3.2 (15 % cardiolipin) there are a broad endothermic event with several maxima that can be seen: the first peak is probably a combination between two transitions with its two maxima at T= 25°C and T= 28°C. The higher-temperature peak at 40°C is broader than the lower-temperature peaks. In the Figure 3.3 (30 % cardiolipin), there are only one broad peak that has two maxima at 33°C and 40°C.

There was no meaning in calculating the enthalpy effects for the peaks in the two figures because the phase transitions could not be separated. The result can be compared with the thermograms for the binary systems in pH 5,5 (fig 2.2 and fig 2.3), and with the result from the sample with lower concentration of cardiolipin (fig

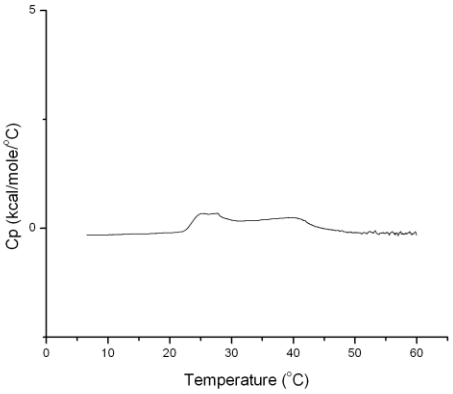

Figure 3.2: 15 mole% cardiolipin in DMPC in pH 5,5

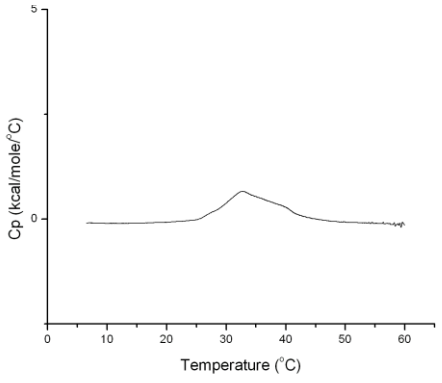

Figure 3.3: 30 mole% cardiolipin in DMPC in pH 5,5

3.1). The first conclusions that could be drawn was that there are no signs of a pre-transition, which occur at a T< 20° C. The peaks at the highest temperatures in the mixture with 15%, T=40°C and 30%, T=40°C cardiolipin, are probably from almost pure cardiolipin phase, because they correspond well with the chain melting transition for pure cardiolipin at the same pH. The peaks that was spotted at T=25°C, 28°C for the 15% mixture of cardiolipin and T=33°C for 30% cardiolipin, are likely to be from a DMPC rich phase. The melting point of a DMPC-rich phase that contain some cardiolipin is expected to be a higher than that of pure DMPC as some cardiolipin with higher melting temperature is mixed into the bilayer. Finally the DSC trace implied that DMPC and cardiolipin are not miscible in the solid phases at this composition and pH.

Table 3: The temperatures and enthalpies for the transitions in cardiolipin/DMPC mixture at pH 5,5.

Cardiolipin in pH 5,5	T (°C)	ΔH (kal/mole)
5%	18	370
	25	4800
15%	25	-
	28	-
	40	-
30%	33	-
	40	-

4.2.2 DMPC-cardiolipin mixtures at pH 9:

As seen in Fig 3.4 the mixture of DMPC/ cardiolipin in the ratio 95:5, has one pre-transition at T_{pr}= 17°C with an enthalpy effect of 410 cal/mole, and one main transition at 25°C with a higher enthalpy effect of 4500 cal/mole. As can be seen from the DSC trace, it clearly resembles that of pure DMPC in water (figure 2.1). The pre-transition is observed also in the presence of cardiolipin, but it is shifted to a higher temperature as well as the main transition. This indicates that the lipids are miscible at this concentration. The broadening of the peak corresponds to ΔT= 4°C.

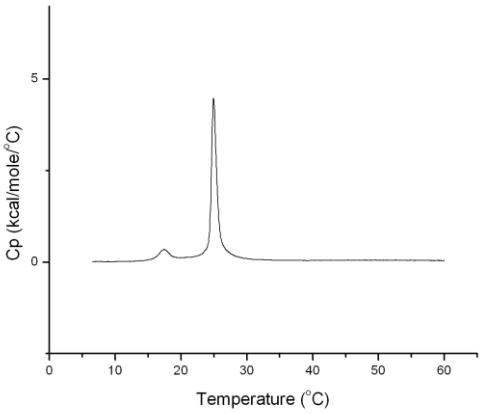

Figure 3.4: 5 mole% cardiolipin in DMPC in pH 9.

Similar results were obtained for the higher cardiolipin concentrations, as shown in Figure 3.5 and 3.6. The melting temperatures increased with increasing amount of cardiolipin, and was measured to T_m= 27°C at 15 mole% cardiolipin and to T_m=29°C for 30 mole% cardiolipin. With higher concentration of cardiolipin the pre-transition disappeared and the peaks was broadened, the ΔT for 15 mole% cardiolipin was measured to ΔT=10°C, and for 30 mole% cardiolipin to ΔT=16°C. The measured transition enthalpies, ΔH_m=7000 cal/mole for 30 mole% cardiolipin and ΔH_m=4800 cal/mole for 15 mole% cardiolipin, were significantly higher compared to the pure DMPC, but still lower than the melting transition enthalpy of pure cardiolipin.

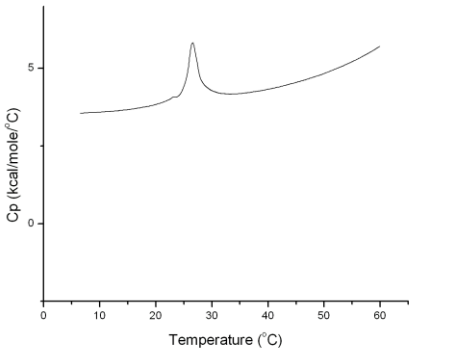

Figure 3.5: 15 mole% cardiolipin in DMPC in pH 9.

Table 4: The temperatures and enthalpies for the transitions in cardiolipin/DMPC mixture at pH 9.

Cardiolipin pH 9	T (°C)	ΔH (kal/mole)
5%	17	410
	25	4500
15%	27	4800
30%	29	7000

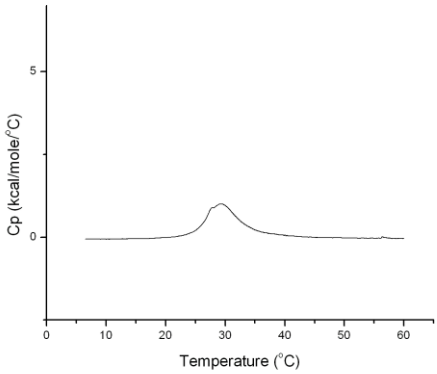

Figure 3.6: 30 mole% cardiolipin in DMPC in pH 9

4.2.3 DMPC-cardiolipin mixtures at pH 7:

When the two lipids DMPC and cardiolipin are mixed with the ration 95:5, as seen in the Fig 3.7, it results in a pre-transition at 18°C with a enthalpy effect of 290 cal/mole and a main transition at 25°C with the enthalpy effect 4400 cal/mole. With low concentrations of cardiolipin the pH nearly have no effect on the miscibility of cardiolipin in the DMPC bilayer. The broadening of the peak corresponds to ΔT= 6°C.

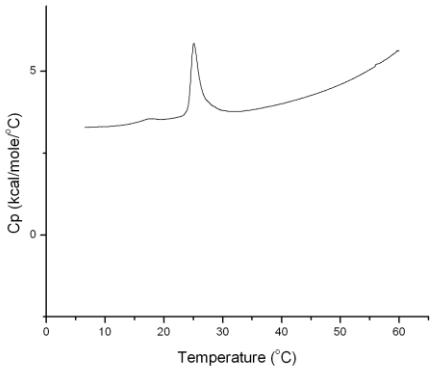

Figure 3.7: 5 mole% cardiolipin in DMPC in pH 7

Like previous scans at pH 5,5 for DMPC/Cardiolipin 85:15, 15 mol% cardiolipin in pH 7 here in Fig 3.8 has several not resolved peaks. The terminal event at 28°C and 37°C can be assumed to be melting of a cardiolipin enriched phase. This lipid mixture has a complex phase behavior, and indicates that the lipids are not miscible in the gel phases.

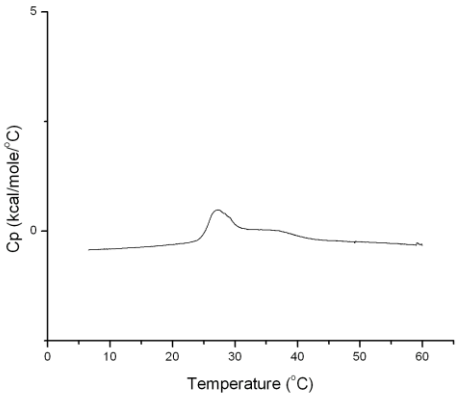

Figure 3.8: 15 mole% cardiolipin in DMPC in pH 7

Table 5: The temperatures and enthalpies for the transitions in cardiolipin/DMPC mixture at pH 7.

Cardiolipin in pH 7	T (°C)	ΔH (kal/mole)
5%	18	290
	25	4400
15%	28	-
	37	-

4.3 Miscibility of CL in a liquid crystalline PC bilayer:

Figure 3.8 and 3.2 imply that 15% cardiolipin is not miscible in the DMPC gel phases at pH 7 and pH 5,5. To explore this result, a similar experiment where DMPC was exchanged with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was performed. DOPC forms a liquid crystalline lamellar phase at all relevant temperatures (melting transition far below 0°C), which means it forms a liquid phase under all the DSC scans (8). DOPC have the same headgroup as DMPC, but the carbon chains are not the same as DMPC (C18:1 instead of C14:0). The hydrophobic thickness in liquid crystalline bilayer expected to be rather similar for DOPC and cardiolipin (C14:0) and therefore do not expect segregation based on their difference in acyl-chain length (13). Cardiolipin was mixed in the same ratios 85:15 as for DMPC and hydrated in the Mes buffer at pH 5,5 see Fig 4.1. Because no transition related to the melting of cardiolipin could be seen it is concluded that cardiolipin is able to dissolve in the DOPC liquid crystalline bilayer.

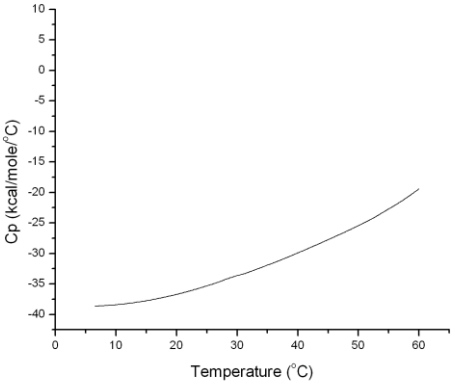

Figure 4.1: 15 mole% cardiolipin in DOPC in pH 5,5*

4.4 metastable phases:

According to literature (12), cardiolipin have a tendency to form metastable phases that are not equilibrium phases. If metastable phases form, one can typically expect that the equilibration procedure and the scanning history affect the phase behavior and thus the phase transitions. Too investigate this, some of the samples with pure cardiolipin were stored in the freezer at ca -10°C, and other samples were stored in a freezer held at -30° C for at least 24 hours. There was a slight difference between the DSC traces obtained for these two samples, see fig 2.4 and fig 5.1. The sample that had been frozen showed a slightly sharper peak than the sample that had been stored in the freezer with higher temperature. When cardiolipin at pH 5,5 is scanned in the DSC the first scan shows a much more defined peak than the following scans see Fig 5.2. Cardiolipin at pH 7 shows an extra peak in the first scans (see fig 2.5 and 5.3), which disappears during the following scans after the sample has been heated and then cooled by the DSC. The last method that was tried was to ~~do~~ run four scans and then have the DSC to thermostat at 60° C for 30 min and after that run another four scans. This was done to see if the phase behavior would change if the sample hade time mix in the liquid state, but there was

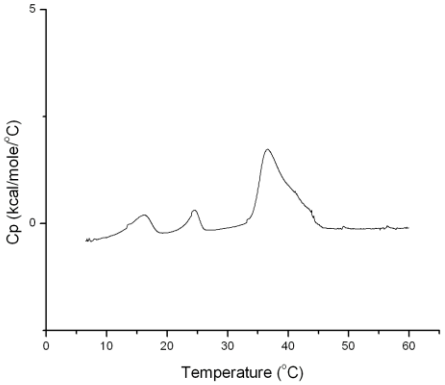

Figure 5.1: Not precooled pure cardiolipin in pH 9

*The y-axel of the scan is a wrong doing of filling the DSC, which does not inflict the result, that there are no transitions.

no different between the two sets of scans. When choosing the scan to show in the report the first scans were always chosen because of cardiolipins metastable behavior.

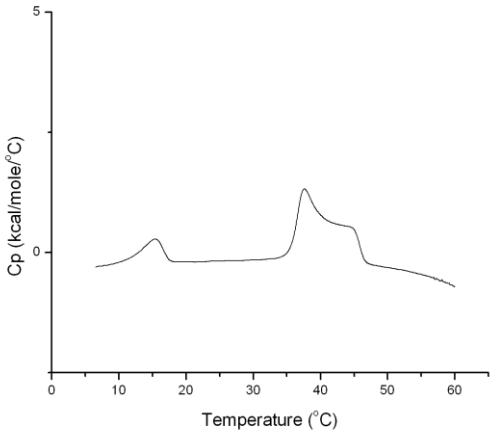

Figure 5.3: The sceond scan of pure cardiolipin in pH 7

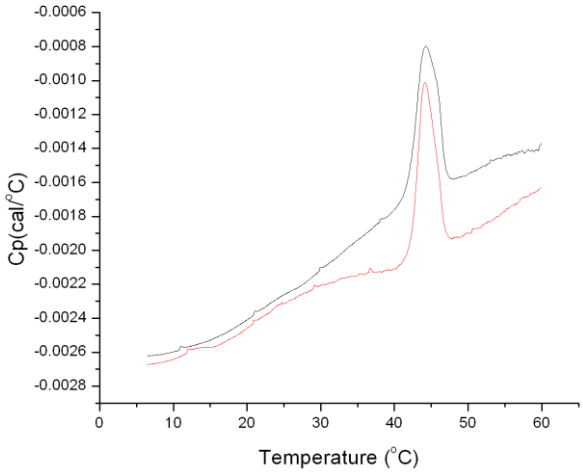

Figure 5.2: Two scans of pure cardiolipin in pH 5,5*

4.5 experimental considerations:

DSC is a method that is very sensitive for contamination in a pure sample. This can be noticed through a broadening of the peaks and sometimes changes in the melting temperatures. DMPC that was used in the experiments have a pre- transition that is sensitive for contamination, because the P_β phase is only formed under pure conditions. In the beginning of the project the pre-transitions was missing in the DSC scans, and the main transition peak was a little broader than expected for a binary lipid-water sample in excess of solution. The source of this contamination was investigated, and the solvent chloroform was suspected of being contaminated. Therefore, a new stock solution was made with a new unopened bottle chloroform. There were also the suspicion that the solvents was not completely dried of after standing in a vacuum oven, so all the samples was left to stand overnight in a evacuator instead. After these changes in the preparation procedure, the where no signs of contaminations in the DSC traces from the DMPC-water samples.

*The y-axel of the scan are not adjusted to the concentration of the sample.

5. Discussion:

5.1 Cardiolipin at different pH:

When looking at pure cardiolipin in pH 5,5 and 9, we can relate the observed differences to difference in cardiolipin headgroup charge, as pKa_2 have been reported to 7,5-9,5 (5). In pH 5,5 cardiolipin should have a negative charge of -1, and in pH 9.0, it should have a charge of -2. When looking at the two thermograms for each of the pH, they indicate quite different phase behavior, and in particular, formation of different solid (gel) phases. Cardiolipin in pH 7 is probably a mix between the cardiolipin species with charge -1 and -2, which can be assumed when comparing the thermograms of pH 7, pH 5,5, and 9. The DSC trace obtained at pH 7 show three transitions similar to the DSC trace obtained at pH 9, and the last peak in pH 7 occur at a temperature that correspond to the T_m for cardiolipin at 5,5. The cardiolipin-water system is a three component system and the DMPC- cardiolipin-water mixture is a four component system.

As can be noticed in the result for pure cardiolipin is that the phase behavior is richer at a high pH. The reason for this is not known, but one can speculate that the packing of crystalline phase is sensitive for intermolecular forces and that a change in charge must affect the packing of the lipids due to a change in headgroup-headgroup repulsion.

There exist only studies of cardiolipin (C14:0) phase behavior. In one study, cardiolipin was studied in a solution with much higher ion strength than used in this study(12), 100mM, and at pH of 7,4 . When comparing the phase behavior from literature and this study (pH 9.0), there is a resemblance. But when comparing these two studies one should consider, the different used ion strength, and the impact the ion strength have on the headgroups charge and indirectly the phase behavior.

5.2 Lipid miscibility:

As seen in the result, the DMPC phase transitions are not affected by the changes in pH 5,5, the same result is expected for pH 9, which means that only cardiolipin is affected when pH is changed. When comparing results obtained for the mixed DMPC/cardiolipin systems, one obvious trend is that for all the three pH, the amount of 5 mole% cardiolipin is always miscible with DMPC. This could be because it's a small amount cardiolipin to dissolve. At higher concentrations of cardiolipin, the solubility of cardiolipin in the DMPC bilayer clearly depends on pH. When looking at the mixed systems, the DSC data imply that cardiolipin is miscible with DMPC at concentrations of 15 mol % and 30 mol% cardiolipin at pH 9. This is not the case for 15 mole% and 30mole% cardiolipin in pH 5,5 and 7, which show clearly different transitions compared to DMPC. The general trend that seems to be indicated is that with higher pH then cardiolipin dissolves itself easier in to the DMPC bilayer. This could be because with higher charge the repulsion between the headgoups are greater, and the will to be more separated from each other .Which could increase the tendency for cardiolipin to dissolve in another bilayer.

When comparing the miscibility for cardiolipin in literature there are studies of related systems to the one used in this study. In the literature they have studied the same cardiolipin (C14:0) as in this study mixed with 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) in a buffer with pH 7,4. The same trend that can be seen in the literature as in this study is that in low concentrations cardiolipin is dissolved. But with increased concentration of cardiolipin the miscibility decreases(14).

5.3 Cardiolipins miscibility in gel and liquid crystalline bilayers:

When 15 mole% cardiolipin was mixed with DMPC, which form a solid gel phase at temperatures below ca 23°C, the cardiolipin is not miscible in the solid DMPC gel phase bilayer at pH 5.5. Top investigate the effect of phase behavior, the ability for cardiolipin to dissolve in a PC liquid crystalline bilayer was studied. In these studies, the lipid DOPC was used in the same buffer at pH 5,5. The thermogram showed no traces of cardiolipin phase transitions, and this implies that cardiolipin was fully dissolved in the liquid crystalline bilayer. So cardiolipin have a higher miscibility in a liquid phased bilayer than a solid bilayer.

5.4 Cardiolipin metastable phases:

The metastability for cardiolipin was a major factor when pure cardiolipin was studied in the DSC. As described in the result, the transition peak for cardiolipin in pH 5,5 was affected and broadened when it went up to 60°C and down again to do a new scan. This could be because cardiolipin has no time to form solid phase during the up and down scan. As also described in the result was the importance of letting cardiolipin be in a low temperature for a longer time so it had time establish a equilibrium state in a solid phase. This has also been shown in the literature(12), that the temperature background of the sample have a major impact on phase behavior. The difference between this study and the literature are that they have very complex protocols for sample preparation, which this study lack but the same phase transitions have been seen at least as well resolved. It should also be mentioned that how the equilibrium behaves depends on the conditions such as ion strength etc, which differs between these studies.

6. Conclusions:

- When pure cardiolipin is solved in the three buffers with pH 5.5, 7 and 9, it has different phase behavior for the different pH. This has to do with the to pKa values that cardiolipin has which give cardiolipin different charge on the pH. At pH 5,5 cardiolipin has a charge of -1, at pH 9 a charge of -2 and at pH7 a mixture between -1 and -2. That pH 7 mixture of the two charges is indicated by that the phase behavior for pH7 is similar to both ph 5,5 and 9.

- It was indicated that cardiolipin is miscible in other bilayers at the three pH 5.5, 7 and 9 with a concentration of 5% cardiolipin. With a concentration of cardiolipin over 5% in pH 5,5 and 7 the miscibility decreased.

- For higher pH as pH 9, cardiolipin was dissolvable in concentrations over 5% cardiolipin. This is probably because of the high charge that cardiolipin has at this pH, which makes the headgroups repel each other and makes it easier for them to dissolve in a other bilayer.

- When 15% cardiolipin was mixed with DOPC, it could be seen in the thermogram that this concentration of cardiolipin was miscible in DOPC at pH 5,5. This indicates that cardiolipin is easier dissolved in a bilayer in a liquid crystalline phase, like DOPC, than a solid bilayer as DMPC has.

- It was also concluded that pure cardiolipin has metastable phases, as mentioned in the literature and that the background-temperature has a big influence on the phase behavior of cardiolipin. It is therefore important to let cardiolipin have time to find a stable state before conducting experiments with it.

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