Student project

Lipids under pH stress!?!

Beate Moeser
Christopher Stowasser

April 2008

Preparatory experiments for the study of the pH-dependence of currents through DOPC/DPPC membranes and the attempt to explain the effect of pH on the phase transition of the lipid mixture.

Contents

1 Introduction and motivation

Biological membranes define cellular boundaries, divide cells into discrete compartments with different compositions of the cytoplasm and organize complex reaction sequences [1]. Selective and regulated ion currents over those barriers are essential for cell-signaling-processes, signal-transduction in the nervous system and many metabolical reactions, which take place at the site of the membrane. Therefore the study of membrane-currents has attracted many scientists and is an important aspect in biological, biochemical, pharmaceutical and medical research.

1.1 Structure of biological membranes

Biological membranes are composed of various amphiphilic lipids and proteins. In most cases the lipids form a bilayer (see figure 1.1) and hence serve as the structure forming unit, in which the proteins are embedded. Some proteins span the lipid bilayer and others are associated to the membrane surface.

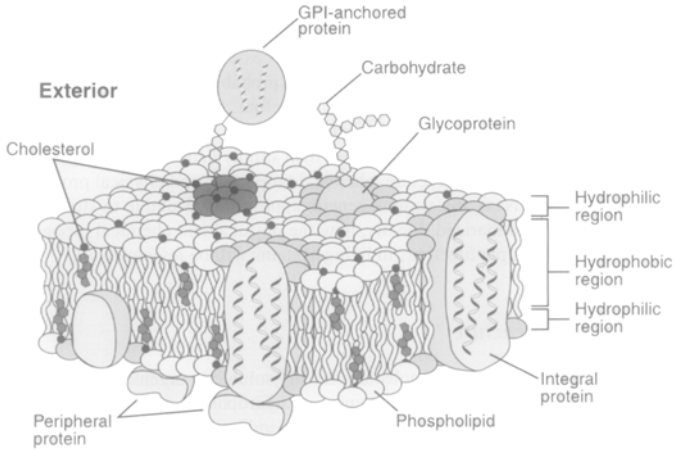


Figure 1.1: The structure forming unit of a biological membrane is the lipid bilayer: The hydrophobic chains of the various lipids form the inside and the polar head groups face the aqueous surrounding. Some proteins span the bilayer, others are only associated to the surface. (figure from: [9])

Depending on thermodynamic variables like e. g. pressure and temperature, the lipid bilayer assumes different phases, whereof the two most important ones are illustrated in figure 1.2. In the solid-ordered phase the hydrophobic chains of the lipids are ordered and tilted and the arrangement of the molecules displays a crystalline order. In the

liquid-disordered phase however the lateral order of the molecules is random, the distance between molecules larger and the hydrophobic chains are disordered. This phase is generally assumed at higher temperatures.

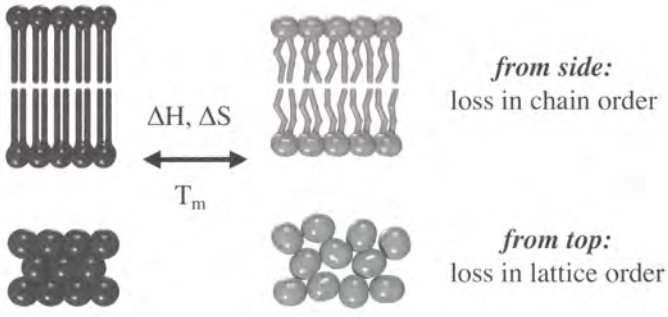


Figure 1.2: Schematic picture of lipid melting from a solid-ordered to a liquid-disordered phase. Top: The order within the lipid chains is lost upon melting. Bottom: The crystalline order of the lipid head groups is also lost and the matrix undergoes a solid-liquid transition. (figure and description from: [2])

In membranes domains of different phases can coexist. Furthermore domains can be found which consist of aggregates and clusters of different lipid- and protein-types. [2]

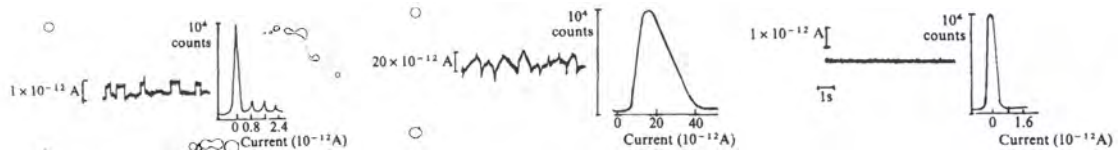
1.2 Permeability of lipid membranes

The hydrophobic inside of the lipid bilayer displays – in principle – a diffusion barrier for ions and all kind of polar molecules. As already mentioned above, discrete, selective and regulated currents however play a crucial role in cell functioning. The occurrence of these currents is often only thought to be due to specialized proteins in the membrane: *Movement of polar compounds and ions across biological membranes requires protein transporters. Some transporters simply facilitate passive diffusion across the membrane from the side with higher concentration to the side with lower. Others bring about active movement of solutes against an electrochemical gradient; such transport must be coupled to a source of metabolic energy.* [1]

Though it could be shown that pure lipid membranes, which do not contain any proteins, exhibit ion currents under certain conditions. [2] [3] These currents have similar characteristics as the currents, that are measured in the presence of proteins. Figure 1.3(a) displays currents through a pure lipid membrane (1,2-Distearoyl-sn-Glycero-3-Phophocholine) and it can be seen, that their amplitudes are quantized (see histogram of measured amplitudes on the right) and that they are of finite duration – both properties, which often are associated with single opening events of protein ion channels.
Figures 1.3(b) and 1.3(c) display measurements at the same membrane, but at different temperatures than in figure 1.3(a). Here none of those characteristic currents are recorded, which nicely illustrates the experimental finding, that the currents mainly occur close to the phase-transition of the lipid bilayer [2] (the currents in figure 1.3(a) were

recorded at the temperature of the transition (59°C)).

These observations *may* be explained by the fact, that changes in the elastic constants of the lipid bilayer (isothermal volume and area compressibility) are proportional to the changes in the heat capacity of the system in the phase transition. This implies that membranes in the transition regime are very compressible and soft [2], which possibly gives rise to transient pores in the lipid membrane.



((a)) at transition ((b)) below transition ((c)) above transition

Figure 1.3: Recorded currents through a pure DSPC membrane at different temperatures and the respective histograms of measured amplitudes: At the temperature of transition current amplitudes are quantized (superposition of amplitudes of the same size). (figure from [3])

1.3 Dependence of the phase transition on pH

Thus the question whether a membrane is permeable or not can be reduced to the question, whether the system is in the temperature regime of the phase transition.

The temperature of the phase transition T_m is the one, at which both phases are in equilibrium, so that their Gibbs free energies are equal. From

$$\Delta G = \Delta H - T_m \Delta S = 0 \tag{1.1}$$

follows

$$T_m = \frac{\Delta H}{\Delta S}, \tag{1.2}$$

meaning that the temperature of transition depends on the difference of enthalpy (ΔH) and difference of entropy (ΔS) between the two phases. Consequently the melting temperature depends on all thermodynamic variables, which contribute to differences in enthalpy between the two phases.

As the polar head groups of the lipids are charged or zwitterionic, the pH is also one of the variables influencing the temperature of transition: Protonation or hydroxylation change the surface charges q of the membrane. As the area per lipid is different in the two phases, this contributes differently to the enthalpies of the two phases and thereby alters ΔH by $\Delta \int \Psi dq$.

1.4 Why should one study the influence of pH on membrane-currents?

In cells and tissues, phosphate and bicarbonate buffer systems maintain intracellular and extracellular fluids at their optimum (physiological) pH, which is usually close to pH 7. [1] Considering this fact the question arises, whether the pH-dependence of the transition temperature (described in the previous section) is of any biological relevance. The answer however is "Yes": Even though the pH in the bulk solution in a cell is kept nearly constant, drastic, short-lasting, local pH-changes can occur at reaction sites. Many receptors in cell-signaling-processes, which are located at the membranes, induce currents through the membrane upon binding of a signaling molecule. Often the signaling molecule is thereby hydrolyzed, so that a proton is released and the pH locally changed. It cannot be excluded, that this change in pH locally shifts the phase transition of the lipids into the present temperature regime, thus enabling ion currents through the membrane. [4]

This example illustrates very nicely, that it is in fact very interesting and relevant to study the influence of pH on currents through pure lipid membranes. The main questions, that are of interest in that context, are:

- Can currents be induced by a change in pH (in a system, which originally was not in the transition)?
- Do existing currents disappear upon a change in pH?
- If currents do not disappear as a result of pH-changes, do any of the currents' characteristics change (amplitude, open-time-distribution)?
- Are the observed currents the same at different pH values as long as the membrane is kept at the corresponding transition-temperature?

The first three questions are more substantial from a biological point of view. The fourth one however is essential in order to understand the mechanism of the channels (is the occurrence of currents in fact only connected to the question, whether the system is in the phase-transition?). Furthermore it is important in order to interpret and support results of experiments addressing the first three questions.

1.5 Measuring of membrane currents with Black Lipid Membrane

A widespread technique to form membranes out of pure lipids and to perform electrical measurements on the membrane is the Black Lipid Membrane (BLM) technique: A Black Lipid Membrane apparatus consists of two separated troughs with a hole covered by a thin teflon film, which has a small aperture (see figure 1.4). The two troughs are filled with buffer solution and lipids are added, so that they form a monolayer on top.

Relative movement of the liquid level with respect to the septum leads to the formation of a lipid double layer in the aperture, if the liquid levels are raised above the aperture.

The two troughs can then be connected with electrodes, so that currents through the formed membrane can be recorded under constant voltage conditions.

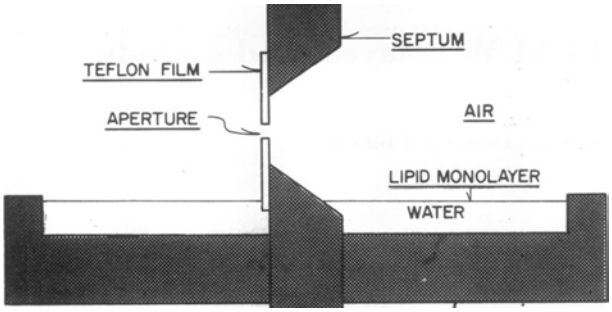


Figure 1.4: The Black Lipid Membrane technique: Lipid monolayers are formed on aqueous solutions in two troughs, which are separated by a septum containing a small aperture in a teflon film. A bilayer is formed in the aperture upon relative movement of the liquid level to the septum. (figure from: [10])

1.6 Auxiliary experiments are needed!

In order to design experiments that address the questions in section 1.4 and in order to interpret the results it is necessary to find out, at which temperature the phase transition lies for different values of pH.

Furthermore it is absolutely essential to ascertain how the pH can be changed and controlled in a BLM experiment.

1.7 Aim of the project/study

In the course of this project these preliminary auxiliary experiments were performed:

- Phase transition temperatures of a DOPC/DPPC[1] (2:1) mixture were determined at different pH by means of differential scanning calorimetry (DSC).
- The change of pH in a BLM experiment after the addition of acids and bases was studied.

Moreover additional experiments were carried out in order to understand the outcome of the experiments mentioned above.

[1]DOPC: 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine
DPPC: 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine

On the basis of the results of those experiments we finally tried to suggest how to approach BLM experiments addressing the main questions in section 1.4 and hope to provide a good groundwork for future studies on the pH-dependence of currents through pure lipid membranes. Besides we aimed to explain the influence of pH on the phase behavior of the DOPC/DPPC (2:1) phosphatidylcholine mixture and thereby hopefully supply a better basis for the analysis of current measurements and the general understanding of how lipid membranes react to pH-changes.

2 Theoretical background

2.1 Lipid phase transitions (and their measuring)

The transition between the solid-ordered phase and the liquid-disordered phase described in section 1.1 goes along with changes in enthalpy, entropy and volume. The higher enthalpy and entropy of the liquid-disordered phase base upon the fact, that some bonds in the fatty acid chains of the lipids are rotated such, that they assume the gauche-conformation (see figure 2.1), which displays a state of higher enthalpy (because of steric repulsion) and entropy (because of degeneracy).

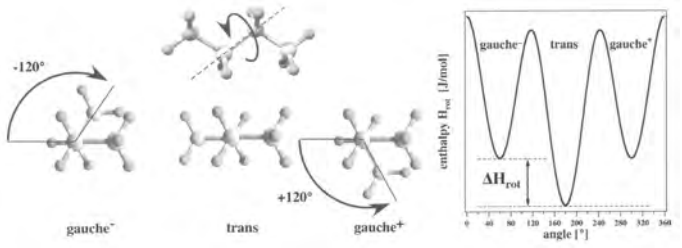


Figure 2.1: The rotation of hydrocarbon chains with at least 4 carbons by multiples of 120°leads to nonequivalent conformations as demonstrated here for butane. Shown are the side view and the view along the central C–C bond. Rotation around the central bond leads to three enthalpy minima: gauche$^-$, trans, and gauche$^+$. The two gauche conformations are identical mirror images. (figure and description from: [2])

The fact, that the enthalpy changes only considerably at the transition, means, that the heat capacity c_p becomes maximal, since the heat capacity at constant pressure is defined as

$$c_p = \left.\frac{\partial H}{\partial T}\right|_p . \tag{2.1}$$

The heat capacity is the energy needed to transfer to a system in order to heat it up by one Kelvin and in the transition a lot of energy is needed for that, since energy first is used for the structural changes. This means, that phase transitions can be detected by measuring the heat capacity. The latter is done with a technique called differential scanning calorimetry (DSC):
A calorimeter consists of two cells, whereas one is filled with the sample solution and the other one with a reference solution. The two cells are heated simultaneously at a constant rate and the electrical power needed to do so is recorded. The power difference

($\mathrm{J\,s^{-1}}$), after normalization by the scanning rate ($\mathrm{K\,s^{-1}}$), is a direct measure of the heat capacity difference between the sample solution and the reference (in $\mathrm{J\,K^{-1}}$) at the respective temperature. [5]

The gel to fluid transition of the lipids used in this project takes place at $-21°\mathrm{C}$ (DOPC) and 42°C (DPPC) [6]. It is expected, that the different lipids do not melt independently, so that the mixture has one phase transition at a temperature between the two values. [2] The heat capacity peak in a DSC-scan of pure lipids is usually very narrow because of a high cooperative unit size – meaning that almost all of the lipids melt simultaneously. In mixtures however the number of lipids melting simultaneously is in general lower, so that phases coexist in a transition regime. The melting profile is broader. [2]

2.2 Head group charge at different pH-values

Figure 2.2 displays the structures of the two lipids used in this study. It can be seen, that they only differ in the hydrophobic hydrocarbon chain, but have the same head group consisting of choline esterified with phosphoglycerine. At neutral pH values the head group carries a negative charge (at the phosphate group) and a positive charge (at the choline group) and therefore does not have a net charge.

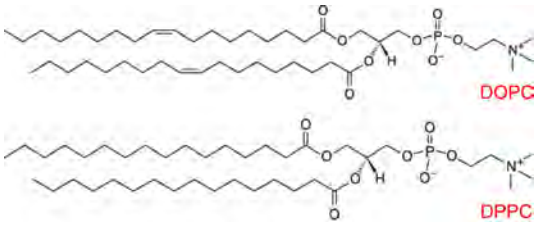


Figure 2.2: structures of DOPC and DPPC: both have a polar head group consisting of choline esterified with phosphoglycerine. DOPC has oleoyl (18:1 cis) and DPPC palmitoyl (16:0) hydrocarbon chains. (structures from: [7])

The phosphate group (PO_4^-) is a classical Brønsted base (A^-) and can be protonated at low pH values according to:

$$AH + H_2O \rightleftharpoons A^- + H_3O^+ \tag{2.2}$$

Whether more of its protonated (AH) or deprotonated form (A^-) is found, depends on its proton binding affinity in the aqueous solution, which can be characterized by the acid dissociation constant:

$$K_a = \frac{[A^-] \cdot [H_3O^+]}{[AH]} \tag{2.3}$$

The smaller the proton binding affinity, the stronger is the acid AH (higher concentration of A^-) and the larger is K_a. Often the pK_a value is used instead of the acid dissociation constant. It is the negative logarithm to the base of 10 of K_a:

$$pK_a = -\log_{10}(K_a) \tag{2.4}$$

Thus the smaller the pK_a the stronger the acid.
Insertion of $[H_3O^+]$ from equation (2.3) into the definition for the pH-value yields:

$$pH = -\log_{10}\left(\left[H_3O^+\right]\right) \tag{2.5}$$

$$= pK_a + \log_{10}\left(\frac{[A^-]}{[AH]}\right) \tag{2.6}$$

Equation (2.6) is called Henderson-Hasselbalch equation. It demonstrates, that the acid is 50 % deprotonated at $pH = pK_a$ and 1 % deprotonated at $pH = pK_a - 2$, respectively 99 % at $pH = pK_a + 2$. The pK_a value of the phosphate group in the lipids lies at ≈ 1.1 [11], so that it is expected, that the phosphate group always is charged at pH values > 3 and that neutralization is observed at lower pH values.

The choline group is not a Brønsted acid (H^+ donator), but can be considered as a Lewis acid (electron pair acceptor). It can in principle stabilize a hydroxide ion and therefore may be neutralized at high pH values.[1]

$$Choline^+ + 2\,H_2O \rightleftharpoons Cholinehydroxide + H_3O^+ \tag{2.7}$$

To sum it up: The head groups are expected to be cations at pH values smaller than one, zwitterions over a large range of pH and possibly anions at high pH. The titration curve of such a lipid is thus – at least at low pH – expected to be similar to the one of e. g. neutral amino acids (see figure 2.3). Figure 2.3 shows that the addition of a base in a pH range of $pK_a \pm 1$ does not considerably change the pH of the solution. In that range the molecule is a buffer. The point of inflection between the two pK_a values is called the isoelectric point pI. It marks the pH at which the molecule is internally neutralized (all molecules are zwitterionic).

$$pI = \frac{pK_{a1} + pK_{a2}}{2} \tag{2.8}$$

[1] According to a paper from T. H. Jukes from 1934 [11] the choline group can not neutralize a hydroxide ion. Fišar however writes [12]: *Over a wide range of pH values, both PC and PE molecules are completely electroneutral as the negative charge of the phosphate group ($pK_{PO_4^-} \leq 1$) is compensated by the positive charge of the choline head ($pK_{NH_3^+} = 11.25$); however, PC and PE become negatively charged at high pH.* It is not clear, whether the stated pK value refers to the choline group of PC or the amino group of PE.

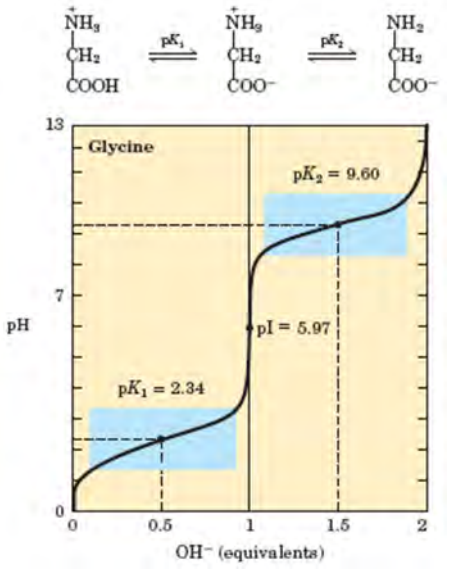


Figure 2.3: Titration of the zwitterionic amino acid glycine with a base. The structures assumed at different pH values are displayed above. In the blue shaded range, glycine is a buffer. The isoelectric point marks the pH at which the molecule is internally neutral. (figure from: [1])

2.3 Our initial hypothesis about the effect of head group charge on the temperature of transition

The polar head group of the lipids is charged at low (and possibly high) pH values and does not carry any net charge in the neutral regime of pH. Therefore we expect, that a change of pH from neutral values into the vicinity of the pK value(s) lowers the temperature of phase transition: *Intuitively one would predict that the melting temperature of a charged lipid membrane is lower than that of a neutral membrane, since the charges on the head groups repel each other, favoring the fluid state with larger area.* [2]

3 Materials and methods

3.1 Lipids

If not specified differently, all experiments were performed with a 2:1 mixture of 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC). DPPC was purchased from Avanti® Polar Lipids, Inc. and DOPC from Sigma-Aldrich™.

3.2 Buffers

If buffers were used, they were prepared according to the so-called "alternatively" recipe generated with the Buffer Calculator by Rob Beynon (http://liv.ac.uk/buffers/buffercalc.html). If not stated particularly, the buffer concentration was 10 mM and the ionic strength 150 mM (adjusted with KCl).

3.3 Differential Scanning Calorimetry

3.3.1 Preparation of samples

The concentration of the lipid-mixture in the DSC-scans was 25 mg/ml. The lipids were weighed in with a precision of 0.1 mg and dissolved in chloroform. After the chloroform evaporated, the sample was put in an exsiccator for at least two hours. Afterwards the correct amount of solvent was added with a micropipette. The lipids were dissolved by shaking with an electrical shaker and – if necessary – by heating up. Finally the sample was put in an vacuum pump for 5 minutes in order to remove air bubbles.

3.3.2 Implementation/Accomplishment of scans

The prepared sample was filled into the sample cell of a VP-DSC instrument (MicroCal™ Inc.) and the reference cell was filled with millipore-water (Barnstead EasyPure RF). Three scans were run in the temperature range between 2°C and 40°C with a scan rate of 5°C per hour (one up-scan, one down-scan and a second up-scan, whereas the third scan was often terminated before it ended).

3.3.3 Analysis of data

Data were analyzed with the software Igor Pro (WaveMetrics, Inc.). The recorded electrical power was transformed into the specific heat capacity of the sample substance

using the scan rate, the absolute mass of sample molecules in the cell and their molar weight. Then a linear baseline was subtracted.

Used molar weights are:

DOPC:	786.150 g/mol	[7]
DPPC:	734.050 g/mol	[7]
HEPES:	238.306 g/mol	[8]

3.4 Black Lipid Membrane

The experiments addressing the pH-control in a BLM-experiment were designed for a BLM-experiment, in which 6 μl of a 25 mg/ml lipid solution (decane/$CHCl_3$/MeOH 7:2:1) are used. The amount of buffer solution (10 mM HEPES with 150 mM KCl) in the troughs is 2 ml in total. The teflon film is prepainted with 5% hexadecane in pentane.

3.5 pH-control

3.5.1 Acids and bases

Experiments were performed with hydrochloric acid (HCl) and sodium hydroxide (NaOH). If needed stock solutions were diluted with millipore water. The volume of the stock solution needed for a volume V_{total} with a concentration $[H_3O^+]_{total}$ was calculated as follows:

$$V_{stock} = \frac{V_{total} \cdot [H_3O^+]_{total}}{[H_3O^+]_{stock}} \tag{3.1}$$

3.5.2 Measurement of pH

pH-measurements were carried out with a "Sen Tix HW" pH-electrode and the pH-meter "pH 538" (WTW GmbH). The electrode is appropriate for measurements in the pH-range from 0 to 14. The pH-meter has a resolution of 0.01. Before a series of measurements the instrument was calibrated with a "conventional two-point calibration" with standard-solutions for pH 4 and pH 7.

4 Results and discussion

4.1 Determination of the phase transition temperature

4.1.1 A typical DSC-scan of the lipid mixture

In figure 4.1 the three DSC-scans of the lipid mixture at pH 5 (acetic acid as buffer) are displayed. They serve to illustrate some common characteristics found in most of the scans:

- The transition temperature lies in general in a range between 20°C and 30°C (here $\approx$ 25°C for up-scans and $\approx$ 24°C for the down-scan) and therefore between the transition temperatures of the pure lipids. This matches our expectations that the lipids do not melt independently (see section 2.1)
- The heat capacity profile is broad (here: FWHM $\approx$ 10°C).
- Heat capacity profiles measured in up-scans match very well. They exhibit a pre-transition at lower temperatures (here $\approx$ 7.5°C).
- In the down-scan the whole profile is shifted to lower temperatures with respect to the up-scans. It displays a higher peak and no pre-transition.

To ensure comparability only up-scans were used in the analysis of our data.

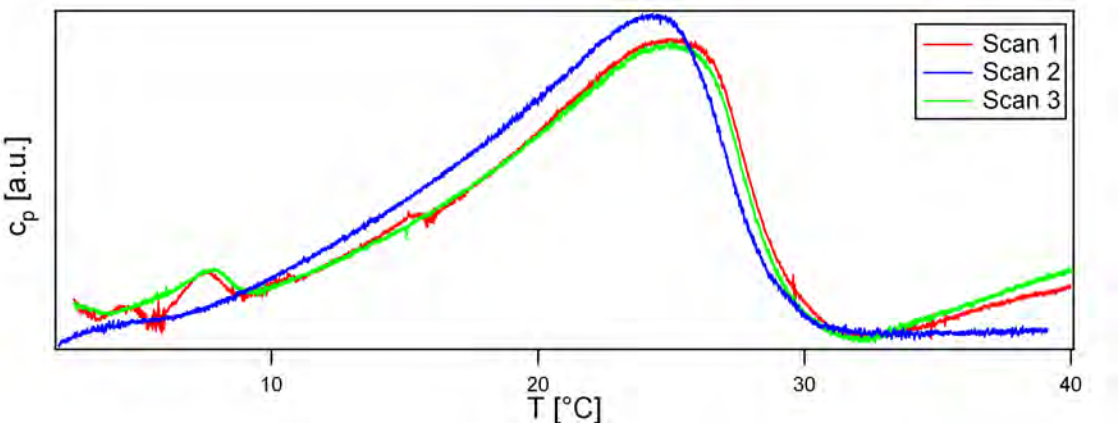


Figure 4.1: The three DSC scans of the lipid mixture at pH 5 (acetic acid as buffer). The transition lies at $\approx$ 24-25°C. Heat capacity profiles measured in up-scans match very well. In the down-scan the whole profile is shifted to lower temperatures, the peak is higher and no pre-transition is observable.

4.1.2 DCS scans at different pH values

We wanted to compare the heat capacity profiles of a lipid mixture at pH 8.5, pH 7 and pH 5. The heat capacity profiles we obtained by DSC are shown in figure 4.2. The experiments were done with the standard lipid mixture in the presence of a buffer corresponding to the adjusted pH. The buffers were chosen as recommended on http://liv.ac.uk/buffers/buffercalc.html. We chose the buffers in a way that the pK_a value of the buffer was as close as possible to our adjusted pH. Therefore we used Tris ($pK_a = 8.06$) at pH 8.5, HEPES ($pK_a = 7.66$) at pH 7 and Acetic Acid ($pK_a = 4.76$) at pH 5.

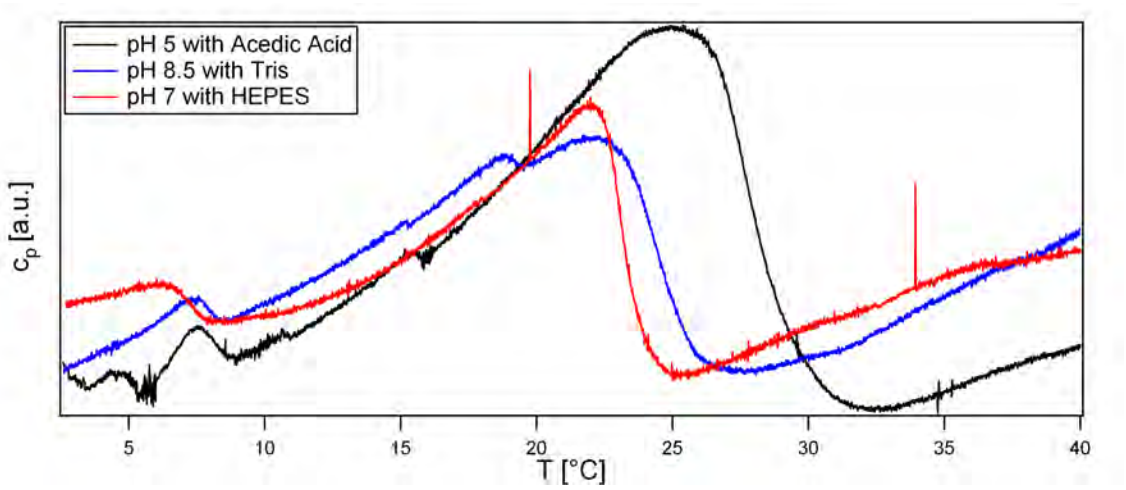


Figure 4.2: Heat capacity profile of the lipid mixture at pH 7 (HEPES), pH 8.5 (Tris), and pH 5 (Acetic Acid). The transition temperatures are: 22°C at pH 7, 22.1°C at pH 8.5 and 25°C at pH 5.

The transition temperatures we found are 22°C at pH 7, 22.1°C at pH 8.5 and 25°C at pH 5. Hence we see no shift of melting temperature at pH 8.5, i. e. for more basic conditions and a shift to higher temperature for pH 5, i. e. for more acidic conditions. The shift of melting temperature to a higher temperature at pH 5 is not in agreement with our idea of electrostatic repulsion (see section 2.3). We expected no shift of the melting transition at all since the pH is still far from the pK_a of the head group of the lipids. We also expected this shift to lower temperatures.

It is difficult to compare currents measured in two independent BLM experiments – i. e. currents through two different membranes. Hence it is reasonable to change the pH during the use of only one lipid membrane. If so, it is not possible to change the buffer for measurements at two different pH values. Therefore only one buffer was used in our subsequent experiments (HEPES).

4.1.3 Heat capacity profile of HEPES

The Scan of HEPES with the normal salt concentration of 150 mM KCl but without any lipids is shown in figure 4.3. The heat capacity profile of HEPES displays no transition in the temperature regime we observed, i. e. from 2°C to 40°C. Therefore it is sufficient for our analysis to substract a fitted straight line from the heat capacity profile instead of substracting the precise heat capacity profile of HEPES.

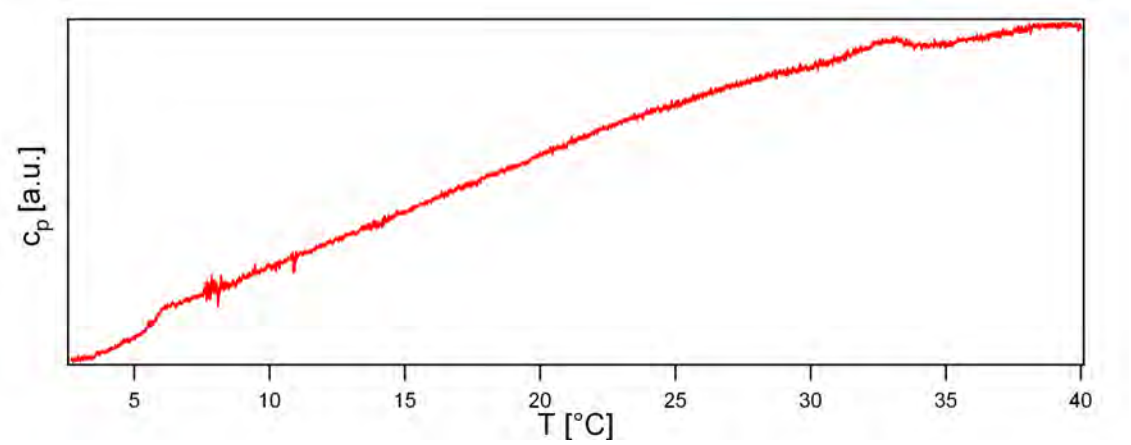


Figure 4.3: The heat capacity profile of HEPES in the observed temperature regime (2°C to 40°C). The profile is nearly a straight line and does not display a peak.

We always used water as a reference. For the later analysis it would have been more convenient, if we used water with 10 mM HEPES and 150 mM KCl. For future experiments we therefore recommend to use as a reference exactly the same solution as the one in which the lipids are dissolved.

4.1.4 Transition temperatures at different pH (with HEPES)

Figure 4.4 displays heat capacity scans of the lipid mixture at pH 1.4, pH 2.8, pH 4.6, pH 7, and pH 11.5. The lipids were dissolved in a solution with HEPES and the pH was then adjusted via addition of HCl or NaOH.

The transition temperature at pH 7 lies at 22.0°C and for all other pH values higher transition temperatures were detected:

- 22.8°C at pH 4.6
- 23.1°C at pH 2.8 (and a shoulder in the profile at $\approx$ 25.2°C)
- 23.9°C at pH 1.4 and a second distinct transition at 29.5°C
- 23.6°C at pH 11.5 (and a shoulder in the profile at $\approx$ 26.1°C)

The lower the pH (starting from seven) the higher is the transition temperature. Shifts in the transition temperature can also be observed at pH values, that are further than 2 values away from the pK value (e. g. at pH 4.6).

The direction of the observed shifts does not correspond to our expectations. Under the assumption that only the net charge of the head groups determines the transition temperature (see section 2.3), the findings have to be interpreted such, that the head groups are neutral at low pH, charged at neutral pH and neutral at high pH. This is contradictory to the expected behavior of phosphatidylcholine (see section 2.2), so that "electrostatics" in the sense described here can be excluded as the single reason for the shift in the transition temperature. Therefore we decided to conduct additional experiments to understand the observations. These experiments are described in the following section.

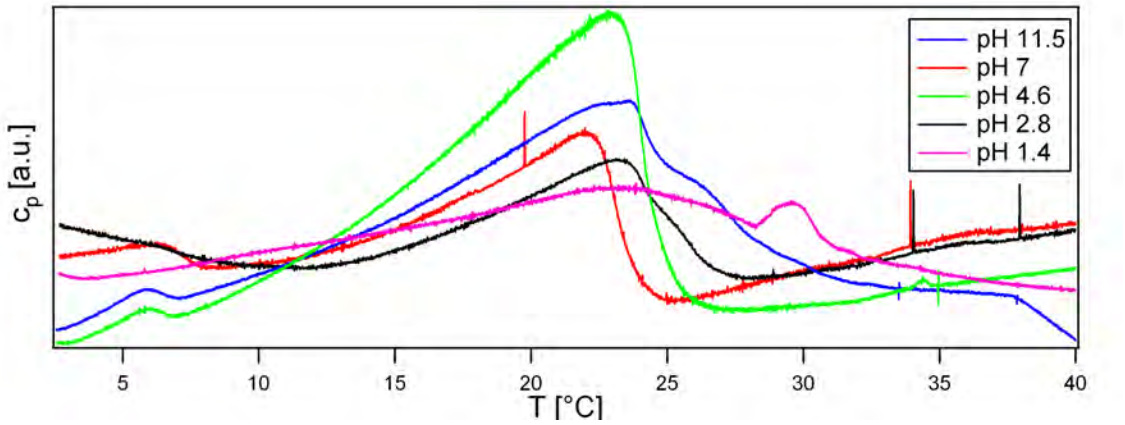


Figure 4.4: Heat capacity profiles of the lipid mixture in HEPES at pH 1.4, pH 2.8, pH 4.6, pH 7, and pH 11.5. The lowest transition temperature can be observed at pH 7. The more acidic the pH the higher is the transition temperature. (Transition temperatures are listed in the text)

4.1.5 Experiments designed to explain the observed direction of the shift

Determination of pK and pI

First we decided to measure a titration curve of DPPC in order to find out precisely at which pH values the head group is charged and where the isoelectric point lies. We were especially interested in the question, whether the choline group gets neutralized at high pH. Furthermore we wanted to support our interpretation that the observed shifts are not due to repulsive effects of net charges:

50 mg DPPC were dissolved in 4 ml 0.1 M HCl. Then 1 M NaOH was added stepwise and the pH value recorded. The lipids did not dissolve completely in the acid. Lipid clumps and flakes could be observed and were still present at higher pH values. In a second series of measurement the change of pH upon addition of the base to 4 ml of pure

0.1 M HCl without lipids was measured. Figure 4.5 shows the results of these measurements. Hardly any differences between the curves can be seen.

The pH of the pure HCl solution was 0.88, which corresponds to a H_3O^+ concentration of 132 mM. After addition of lipids, the pH was 0.90 ($\hat{=}$ 126 mM H_3O^+). This means that $6/132 \approx 5\,\%$ of the acid was needed to protonate the lipids in the beginning. Thus during the first measurement only 95 % of the amount of the present HCl was titrated, whereas in the second measurement 100 % was titrated. Therefore it would have been correct to perform the second titration with 4 ml of HCl with a 5% lower concentration. Then a subtraction of the amount of NaOH needed in the second experiment from the amount used in the first experiment for each pH value, would have yielded the amount of NaOH needed to deprotonate the lipids only – i. e. the titration curve of DPPC.

This was however not done, meaning that we failed to measure the titration curve of DPPC because of incorrect planning of the experiment. As this mistake was detected too late, we could not repeat the measurement. Furthermore the obtained data indicate, that the experiment failed for additional reasons: No considerable difference between the two measurements can be observed, in other words no effect of the lipids was found. This may be due to a too low concentration of (dissolved) lipids. Moreover it is questionable whether the pH measurement and the determination of volumes of acid and base with the micropipettes were exact enough.

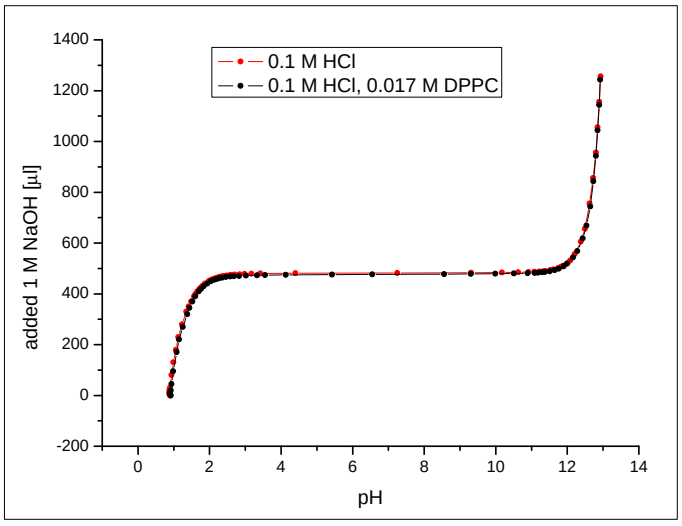


Figure 4.5: Titration curve of 4 ml 0.1 M HCl with 0.017 M DPPC (black) and of 4 ml 0.1 M HCl (red). No considerable differences can be seen.

Analysis of transition profile

In section 4.1.4 it was described, that the position of the peak of the heat capacity profiles depends on the pH. This is usually denoted as being the temperature of transition in our report. It can however additionally be seen (see figure 4.4), that a change in pH also induces changes in the shape of the broad profile of the transition, which also was already indicated before:

- At pH 7 and pH 4.6 the profile consists of only one distinct peak, whereas the slope of the ascent is smaller than the one of the descent.
- At pH 2.8 and pH 11.5 the profile displays a shoulder in the descent of the peak.
- At pH 1.4 the profile is considerably broader than the other profiles and a distinct second peak can be seen in the descent at a higher temperature than the shoulders at pH 2.8 and pH 11.5.

These findings indicate that a shift in pH does not only change the temperature of transition but also the process of melting itself:
A broader profile indicates, that less lipids melt simultaneously (smaller cooperative unit size). This *may* be due to

- altered interactions between lipids in the fluid and in the gel phase or between the two kinds of lipids.
- a smaller vesicular size. (the lateral pressure is different in inner and outer layers of a curved membrane, so that the transition temperature of the different layers is different and the profile broadened. [2])

A dispartment into separate peaks may imply that changes in local or macroscopic curvature occur. This has been observed for charged lipids. As the elastic constants of a membrane are highest in the chain melting transitions, it is likely that curvature transitions are induced by melting. [2]

Irreversible changes under very acidic conditions?

To check for irreversible changes of the lipid mixture when they are exposed to very low pH values we used the following experiment:

We prepared two lipid mixtures under the same conditions in millipore water (without the buffer HEPES and without KCl). We titrated one of them to a very low pH value (pH 1.08) and left the acidic lipid mixture rest in the fridge for approximately 12 hours. Then we titrated the mixture back to pH 6.9 and determined the heat capacity profile via DSC. To the second lipid mixture we added the same amount of HCl and NaOH as we did for the first mixture but here we mixed the HCl and NaOH before they came in contact with the lipids. After the addition the lipid mixture was at pH 7.92. Hence the

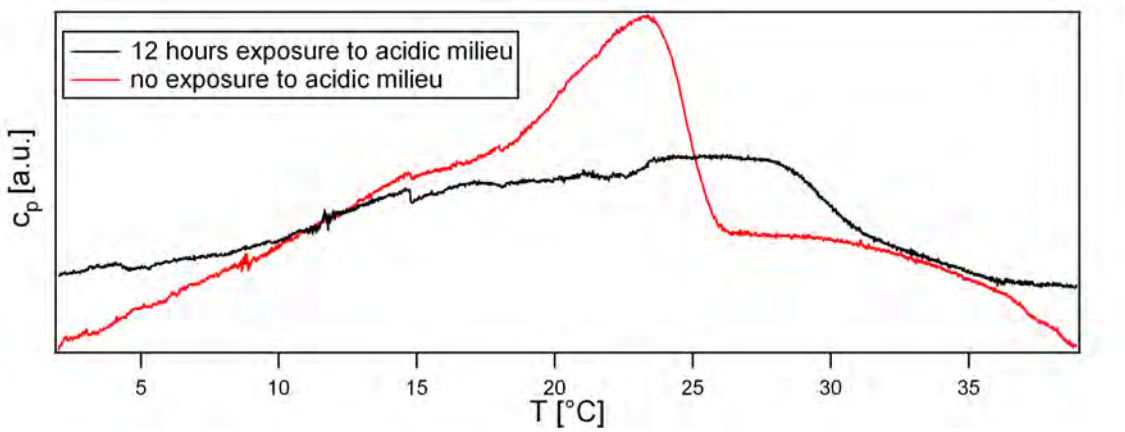


Figure 4.6: Black: Heat capacity profile of a lipid mixture, which was exposed to acidic conditions for 12 h and then titrated back to pH 6.9 with a base. Red: Heat capacity profile of a lipid mixture to which the same amount of acid and base were added (but already mixed). The profiles look different, which indicates, that irreversible changes take place.

second lipid mixture was not exposed to acidic conditions but was mixed with approximately the same amount of acid and base.

The heat capacity profiles are shown in figure 4.6. The lipid mixture that was not exposed to acidic conditions displays a heat capacity profile approximately like the ones we have observed so far, i. e. a profile with a clear melting transition around 22-23°C. In contrary the lipid mixture that was exposed to acidic conditions for about 12 hours shows a very broad profile with hardly any peak noticeable.

To strengthen our result more measurements would be necessary. Nevertheless we cannot exclude the possibility of irreversible changes of the lipid mixture at low pH values.

4.2 pH-control in the BLM-experiment

Our BLM experimental setup is unsuitable for a pH measurement during the experiment. The two troughs left and right of the teflon film are too small for the electrode of our pH-meter. This was the motivation for an experiment where we tried to simulate the BLM experimental conditions in a separate and bigger trough so that we could measure and adjust the pH. We aimed to create a calibration curve from which the needed volume of acid for a desired pH value in a BLM experiment could be read off.

We used the components listed in section 3.4 that are normally used in a BLM experiment. The mixture was titrated from a neutral to an acidic pH and vice versa as shown in figure 4.7. The slope of the titration with the base at low pH is much steeper than the slope of the titration with acid. Hence we needed less base to reach neutral pH

compared to the amount of acid we used in the former case.
A similar observation was reported from a BLM experiment the master student Kasia Wodzinska conducted: During recordings of currents at pH 7 with HEPES as a buffer 120 μl 1 M HCl were added to lower the pH. After further recordings 120 μl 1 M NaOH were added to check for reversibility. After termination of measurements the pH of the solution was checked and found to be 11.1.

In theory the pH change due to addition of a certain amount of acid can be reversed by addition of the same amount of a base of the same concentration. Our measurements however do not affirm those expectations.

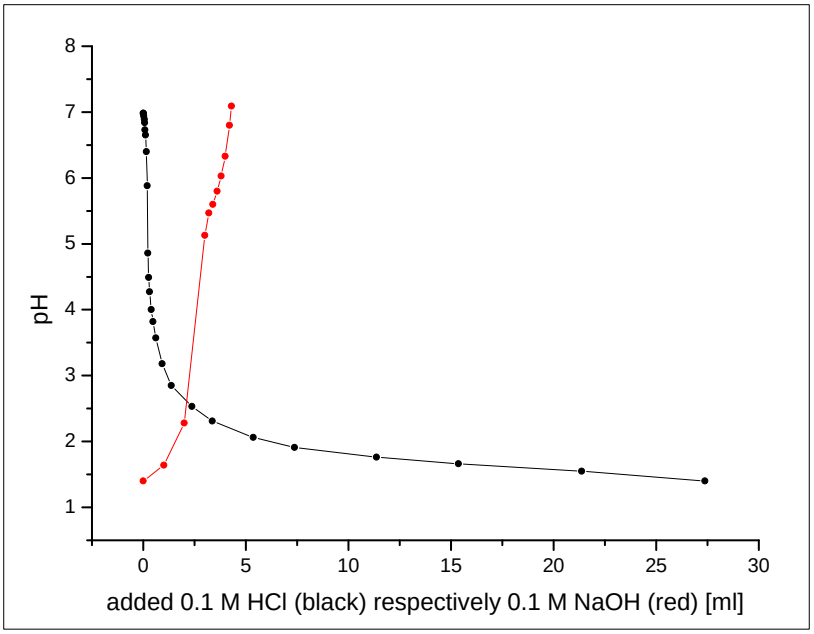


Figure 4.7: pH value of the BLM ingredients after addition of 0.1 M HCl starting from pH 7 (black), respectively after addition of 0.1 M NaOH starting from pH 1.4 (red). Less NaOH is needed to change the pH from 1.4 to 7, than HCl is needed to cause a decrease in pH from 7 to 1.4.

First we assumed the differences were due to effects of the lipids. We used 12 μl of a lipid mixture with a concentration of 25 $\frac{mg}{ml}$. The lipids were dissolved in 4 ml of KCl solution (150mM) with HEPES (10mM). We excluded an effect of the lipids since the concentration of lipids in our BLM simulation was very small:

$$0.012\,ml \cdot 25\,\frac{mg}{ml} \cdot (4\,ml)^{-1} \cdot (768,78\,\frac{g}{mol})^{-1} = 9.75 \cdot 10^{-7}\,\frac{mol}{l} \tag{4.1}$$

As a second attempt to explain the titration we checked the concentrations of the acid (0.1 M HCl) and base (0.1 M NaOH) which were used to titrate the lipid mixture. We measured pH 1.04 for what was supposed to be the 0.1 M HCl and pH 12.85 for what was supposed to be the 0.1 M NaOH. Then we mixed these in equal shares and measured pH 12.08. A theoretical calculation using the same pH values yielded the following result:

$$[H^+]_{HCl} = 10^{-1.04}\frac{mol}{l} = 0.0912\frac{mol}{l} \tag{4.2}$$

$$[OH^-]_{NaOH} = 10^{14-12.85}\frac{mol}{l} = 0.0708\frac{mol}{l} \tag{4.3}$$

with

$$\begin{aligned} pH + pOH &= 14 \\ \Leftrightarrow lg[OH^-] &= 14 - pH \\ \Leftrightarrow [OH^-] &= 10^{14-pH} \end{aligned} \tag{4.4}$$

equal shares

$$\begin{aligned} \Rightarrow [H^+]_{mixture} &= \frac{[H^+]_{HCl} - [OH^-]_{NaOH}}{2} \\ &= \frac{0.0912\frac{mol}{l} - 0.0708\frac{mol}{l}}{2} \\ &= 0.0102\frac{mol}{l} \end{aligned} \tag{4.5}$$

We can neglect the dissociation of the water in the solvent and say, that a surplus of $0.0102\frac{mol}{l}$ of free hydrogen ions is present in the mixture. The pH of this solution is:

$$pH_{mixture} = -lg[0.0102] = 2.0 \tag{4.6}$$

This led us to the following conclusions:

1. Even very small differences in concentration of acid and base result in a significant change of the desired pH.
2. Non-avoidable mistakes, e. g. during pipetting, result also in a significant change of the desired pH – that apparently happened during our measurement. Theoretically we expected pH 2.0, but we measured pH 12.08.

We therefore strongly recommend a pH measurement during the BLM experiment, e. g. with a smaller electrode.

5 Conclusions

5.1 Preparatory experiments for BLM experiments

The first aim of our project was to perform auxiliary experiments for BLM experiments addressing the effect of pH on currents through the 2:1 DOPC/DPPC lipid mixture.

5.1.1 The mixture is appropriate

As our first but essential finding we can state that the lipid mixture undergoes a phase transition at temperatures close to room temperature. Secondly we proved, that a change of pH can result in a change of the heat capacity profile of the lipid mixture. It is therefore reasonable to use the lipid mixture DOPC/DPPC in a BLM experiment, which is used to investigate the pH-dependence of currents through pure lipid membranes.

5.1.2 The pH control is difficult

We found out, that control and adjustment of the pH during a BLM experiment by defined addition of acid or base, enables us only to make qualitative statements. Another opportunity is to measure the pH before and after the BLM experiment by taking the solution out of the setup. Then however no experiments addressing reversibility can be performed. For quantitative analyses a pH measurement in the experiment is unavoidable.

5.1.3 We have ideas about how to approach BLM experiments

On the basis of our measurements (see figure 4.4) we are now able to suggest how to set temperature and pH in a BLM experiment in order to address the questions, which were stated in section 1.4:

- **Can currents be induced by a change in pH (in a system, which originally was not in the transition)?**
  In order to answer this question, it is e. g. reasonable to start recordings at pH 7 and 25°C. The membrane is not in the transition regime and the likelihood to observe currents is accordingly very low. Addition of acid or base should induce the phase transition and therefore increase the likelihood for pore formation.

- **Do existing currents disappear upon a change in pH?**
  This question can for example be approached by starting with the recordings at 25°C and a very low pH. Then a base is added. Thus such an experiment also

serves as a test for reversibility of the observations in the experiment proposed above.

If currents do not disappear as a result of pH-changes, do any of the currents' characteristics change (amplitude, open-time-distribution)?
Possibly changes in characteristics can already be observed in the experiment described above. If not, a more promising experiment would be to measure currents at e. g. 22°C and different pH. According to the heat capacity profiles, it is not expected, that currents, which occur at pH 7 and 22°C, disappear upon a change of pH. At 22°C the membrane is at all measured values of pH in the phase transition regime. The heat capacity is however not highest at 22°C for pH values unequal 7.

- **Are the observed currents the same at different pH-values as long as the membrane is kept at the corresponding transition-temperature?**
  Our measurements revealed that it is hard to define a single exact transition temperature for each pH value, since the heat capacity profiles change shape with pH. Therefore this question can not be approached in its initial meaning. Nevertheless it might be interesting to compare currents recorded at 22°C and pH 7 with currents e. g. recorded at 29.5°C and pH 1.4.

5.1.4 Prospects

In the laboratory BLM experiments at different pH values are already performed. As an example we present results of the master student Kasia Wodzinska (see figure 5.1). Even though she used a 2:1 mixture of DLPC/DMPC[1], the measurements can serve as a prospect for what can be measured with the DOPC/DPPC mixture. All the lipids belong to the group of Phosphatidylcholines and therefore have the same head group.

In the DLPC/DSPC mixture a lowering of pH from seven to two results in a shift of the heat capacity profile to higher temperatures ($\Delta T_m \approx 9°C$). In current measurements at 19°C and pH 7 no currents were observed. At pH 2 single current events were detected at 19°C in the same membrane. Those observations are in agreement with the fact, that currents most likely occur, when the membrane is in the phase transition. We expect similar observations for the DOPC/DPPC mixture at 25°C.

[1] DLPC: 1,2-Dilauroyl-sn-Glycero-3-Phosphocholine
DMPC: 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine

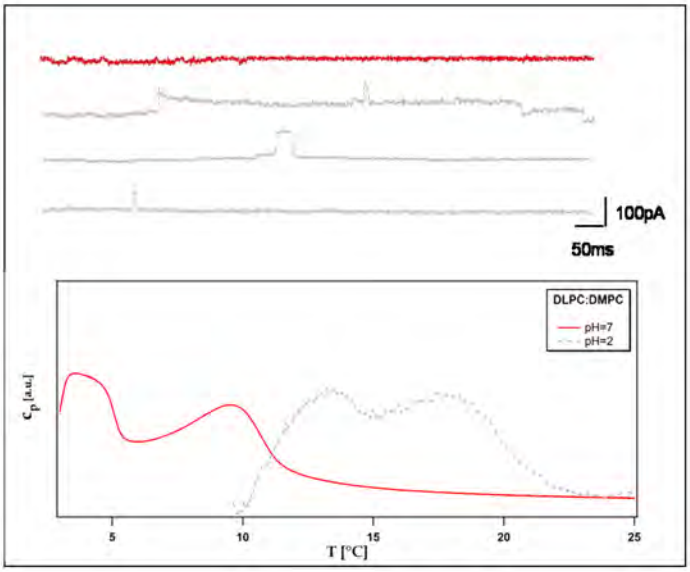


Figure 5.1: Bottom: Heat capacity profile of a DLPC:DMPC 2:1 mixture (scaled) at pH 7 (red) and pH 2 (grey). Top: Corresponding current traces (recorded at 19°C with membrane potential 90 mV).

5.2 Influence of pH on phase transition

It was not planned from the beginning to investigate the influence of pH on the phase transition of the lipid mixture. We thought it could completely be explained by the theory described in section 2.3, which is repeated here:

The lipids do not carry a net charge at neutral pH. When the pH is close to the pK value of one of the charged groups in the head group the respective charge becomes neutralized and the head group carries a net charge. Due to this charge the head groups repel each other and favor the liquid-disordered phase, in which the single lipid molecules are further apart. This means, that lowering or rising the pH (from seven) to the pK values leads to a decrease in melting temperature.

5.2.1 Our initial theory was too simple

Our experiments revealed, that a change in pH manifests itself in a shift of melting temperature to higher temperatures and in a modification of the shape of the profile. Both observations are not be explained by our initial hypothesis.

Alone the fact that the shape of the profiles changes with pH implies that this hypothesis was too simple to describe the seemingly more complex procedures on the surface

of lipid membranes. Structural changes of the lipid formations may be responsible for the observed melting temperature shifts. A non-zero net charge of the lipid head groups may not only result in facilitating the melting process, but also (regarding to our results even more) in a change of structure, e. g. radius of curvature of the lipid formations. Furthermore it is possible that different mechanisms hold for different pH conditions.

Even though experimental results do not significantly support the irreversible change of lipids under acidic conditions, this effect cannot be excluded.

5.2.2 A further hypotheses worth to follow ...

Our results strongly indicate, that the effect of pH can not be due to repulsive effects of net charges between the head groups. This however does not mean, that head group charges can be excluded as a reason for the experimental findings. It seems rather necessary to include dipole moments into consideration when dealing with zwitterionic head groups: The area per lipid in a solid-ordered DPPC membrane is $0.747\,\mathrm{nm}^2$ [2], meaning that the distance between the head groups is approximately $4\,\text{Å}$ and therefore of the order of the distance between the two charges within the lipids. This distance is approximately $6.5\,\text{Å}$ (if angles are not taken into account) as shown in table 5.1. Hence it can not be excluded, that there also is an independent individual interaction between the positive and the negative charges of neighboring head groups. Depending on the relative orientation of the head groups, even stronger repulsions than between single-charged molecules are supposable. Träuble and Eibl observed that the transition temperature of DPPC rises with decreasing pH and ascribe it to the mentioned effect [13].

If these considerations are carried on to an extreme in a *Gedankenexperiment*, one can say, that the electrostatic repulsion between zwitterions is larger than between single-charged ions. Then all observed shifts of the melting temperature would be explained. It should however be considered, that – depending on the charges – structures of different curvature and different orientation of the dipoles may be formed. This might explain different shapes of profiles.

In our mind, this approach is quite promising.

Table 5.1: Bond lengths between the choline and the phosphate group.

bond	**length [Å]**
C–C	1.35 [14]
C–N	1.76 [14]
C–O	1.79 [14]
P–O	1.50 [15]
	6.40

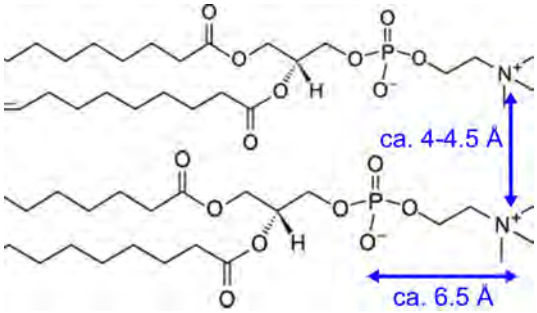


Figure 5.2: The distance between the charges of two neighboring head groups is of the same order than the distance between the charges within a head group.

5.3 Interesting, but not accomplished . . .

During our experiments we often liked to conduct further measurements. Due to limited time we were sometimes not able to do so. Here are some of our ideas:

- More measurements at higher pH.
- DSC scans of single lipids (to exclude possible effects of mixing).
- More measurements in general to statistically support our results.
- Investigation of lipids with other head groups.

5.4 Last but not least . . .

We hope, that we managed to provide a good groundwork for future BLM experiments regarding the pH and helped to understand the pH-dependence of the phase transition. Hopefully we could make the topic more interesting and thereby inspired to think about it (or even study it experimentally . . .).

We would like to thank especially Kasia Wodzinska for helping and guiding us throughout the whole project, and furthermore Thomas Heimburg for inspiring us to work on that topic and for providing us with the theoretical background. Also we would like to thank the members of the Membrane Biophysics Group for practical help whenever it was necessary.

Bibliography

[1] Nelson D., Cox M.: Lehninger Principles of Biochemistry; 4th edition, Palgrave Macmillan, 2004

[2] Heimburg T.: Thermal Biophysics of Membranes; Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2007

[3] Antonov V., Petrov V., Molnar A., Predvoditelev D., Ivanov A.: The appearance of single-ion channels in unmodified lipid bilayer membranes at the phase transition temperature; Nature Vol. 283, 1980

[4] Kaufmann K.: Protron Transport; Caruaru, 1989 (http://membranes.nbi.dk/Kaufmann/ (30.03.2008))

[5] Jelesarov I., Bosshard H.: Isothermal titration calorimetry and differential scanning calorimetry as complimentary tools to investigate the energetics of biomolecular recognition; Journal of molecular recognition, 1999

[6] Lipidat Database (http://www.caffreylabs.ul.ie/OldLipidat/search.stm (26.03.2008))

[7] Avanti Polar Lipids, Inc. (http://www.avantilipids.com/ (30.03.2008))

[8] Wikipedia (http://de.wikipedia.org/wiki/HEPES (10.03.2008))

[9] Vance D., Vance J.: Biochemistry of Lipids, Lipoproteins and Membranes; 4th edition, Elsevier, 2002

[10] Montal M., Mueller P.: Formation of Bimolecular Membranes from Lipid Monolayers and a Study of Their Electrical Properties; Proc. Nat. Acad. Sci. USA, 1972

[11] Jukes T.: The electrometric titration of lecithin and cephalin; The journal of biological chemistry, 1934

[12] Fišar Z.: Interactions Between Tricyclic Antidepressants and Phospholipid Bilayer Membranes; Gen. Physiol. Biophys. 24, 161–180, 2005

[13] Träuble H., Eibl H.: Electrostatic Effects on Lipid Phase Transitions: Membrane Structure and Ionic Environment; Proc. Nat. Acad. Sci. USA Vol. 71, No. 1, pp. 214-219, January 1974

[14] http://en.wikipedia.org/wiki/bond_length (08.04.2008)

[15] http://deposit.ddb.de/cgi-bin/dokserv?idn (08.04.2008)