Isothermal Titration Calorimetry and Differential Scanning Calorimetry of DPPC membranes and ethanol

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Abstract

We use isothermal titration calorimetry and differential scanning calorimetry to examine the effects of ethanol on the melting transitions of DPPC membranes.

We find a lowering of melting temperature of -1.97 $\frac{K \cdot l}{mol}$ by the ITC experiment and -1.47 $\frac{K \cdot l}{mol}$ by the DSC.

1 Introduction

This is an investigation into the thermodynamic properties of lipid membranes. More precisely their thermodynamic properties close to the transition between gel and liquid crystal phases, a point where the thermodynamic properties can play an important role for the mechanical properties of the membranes.

What role the mechanics of membranes play in living organisms, particularly in nervous system, is a hotly debated subject.

2 Experiment

2.1 Isothermal Titration Calorimetry (ITC)

Imagine, if you like, two small containers placed inside a big isolating box. In one of the containers there is water, in the other a test sample. The two containers is continually cooled by a stream of dry air, and heated by a small mechanical device. Between the two containers is a very fine thermometer, capable of registering slight differences in temperature. This thermometer is connected to the heating device, which adjustes the amount of heat sent to the sample container. The difference in heating is registered by a computer(see figure 1 and 2)

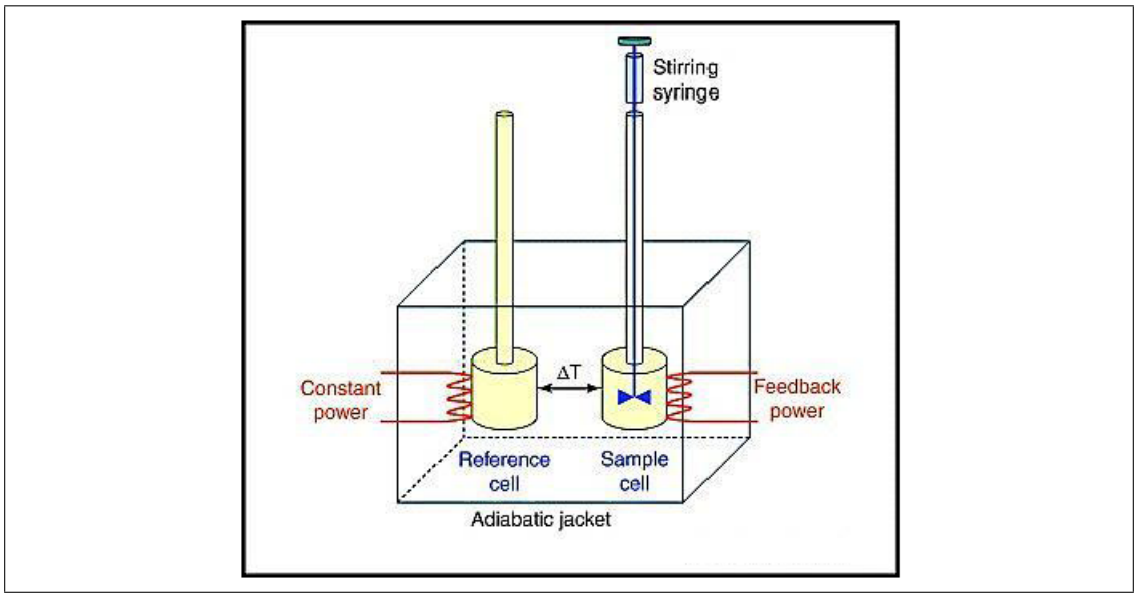


Figure 1: *Schema of a titration calorimeter. The feedback power is varied to keep* ΔT *as close to zero as possible*

Now if a chemical process in the test containers gives a contribution to it's heat, we measure the amount by calculating how much more or less heat was added in order to keep the two containers at the same temperature.

We fill a special made syringe with a tiny amount of another chemical. We insert the tip of this syringe into the test container. The syringe has a fan like blade at the tip. The ITC machine grabs onto the syringe with a robot arm, and rotates it, thereby stirring the cell with the blade. The arm then gradually, in small controlled squirts injects a fixed amount of chemical from the syringe into the cell.

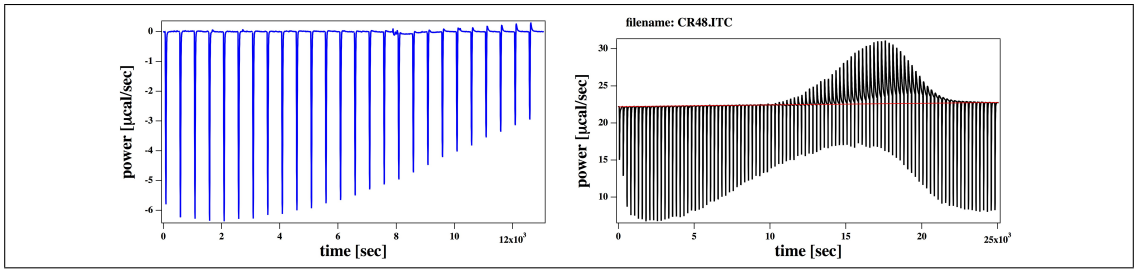


Figure 2: *Example of ITC data. On the left ethanol is injected into water. Each injection releases an amount of heat, stemming from the ethanol being diluted. This causes the temperature to rise(very very slightly), which is negated by the heating for the cell being lowered, which is what we can see. On the right, quite a few more injections are made into water containing DPPC membranes. In the middle, the melting transition is clearly visible*

2.2 Differential Scanning Calorimetry (DSC)

Now picture almost the same machine as for the titration calorimetry. Only, now there is no syringe, only one cell containing the sample and one with water. Both are now heated, or cooled so their temperarues change. They are, however, heated in such a way, that they both have the same temperature throughout the experiment. The data then is the difference in heating between the two cells. If the change in temperature now causes a reaction in the sample cell, either exothermic or endothermic, we will be able to see this from the heating data.

2.3 Measuring Melting

In this particular experiment, we are measuring the melting temperature of certain membranes. How do we do that? Well, phase transitions, like melting, generally

either requires or releases a great deal of energy. When the membranes melt, we will therefore be able to see a shift in the heat transferred to the cell, because some of the heat is absorbed in the melting transition.

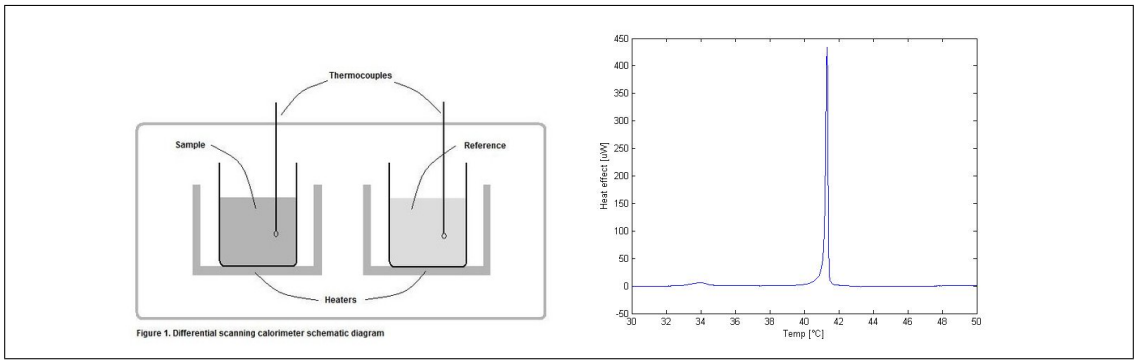


Figure 3: *Schematic of a DSC instrument and an example of DSC data. Here we can see DPPC membranes plus some ethanol melting around 40 degrees which requires the DSC to heat faster in order to keep the temperature rising at the same ratel. It is very straightforward to recalculate the heating effect directly to the specific heat capacity of the membrane.*

3 Lipids

A lipid is a biological molecule which is hydrophobic (repulsed from water) in one place and hydrophilic (attracted to water) in another place. This will mean, when many lipids are together in water, they will tend to cluster so they can turn their hydrophobic parts towards each other, and hydrophilic parts towards the water surrounding them.

There are many ways the lipids can form such clusters. Here we will only look at one: A membrane. A membrane is like a sandwich with two sheets of lipids, all pointing their hydrophoboc ends inwards, and hydrophilic sides out. So together they form a sheet with only hydrophilic sides. On these sides, there then can be as many other sheets, with the hydrophilic side pointing (Like putting extra pieces of bread inside a sandwich). The most basic membrane, with only two lipid sheets is known as a bilayer(figure 5).

The membrane can then take on a variety of shapes, from the cellwalls in a bacteria to huge bubble skins. Most importantly, the cells of humans and other animals are made with a lipid membrane shell. This includes the nervecells, so actually nerves are long wires of lipid membranes.

Lipids mainly consist of hydrocarbon chains. These chains will usually have a certain configuration which has least free energy. At higher temperatures, the

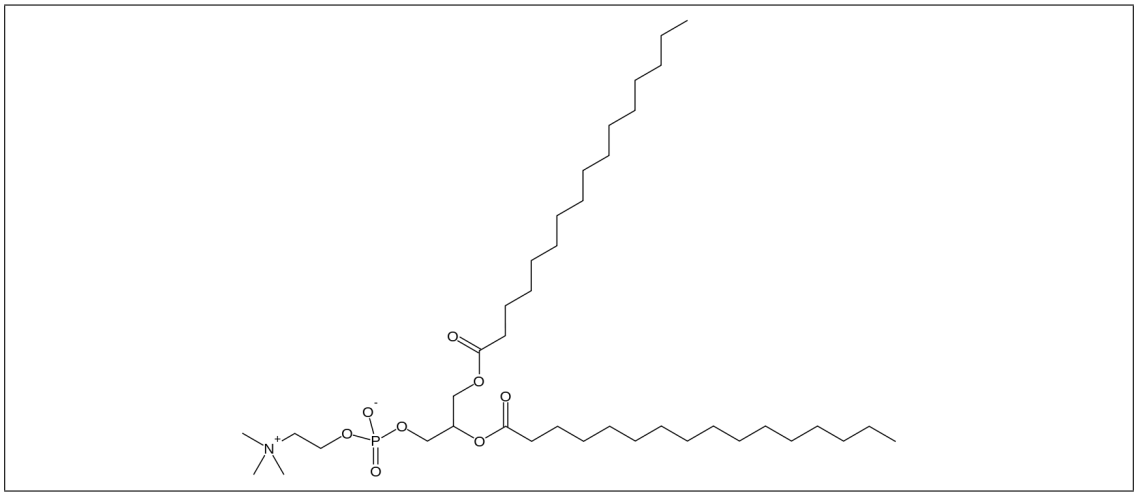


Figure 4: *Structure of a DPPC (short for Dipalmitoylphosphatidylcholine) lipid, the sort I am using for this experiment. The two 'legs' are hydrophobic and the 'head' is hydrophilic.*

chains can take on numerous configurations. We will here call membranes, consisting of lipids with their chains in the least-energy configuration 'gels' and membranes of lipids with their chains shifting shape freely 'liquid'.(figure 6).

Despite the name, liquid membranes can still be shaped just as gel membranes, they are therefore also called 'liquid crystals', but we will just stick with liquid here. Liquid membranes will in general be larger than the gel membranes (It takes more space to have a flailing chain than a nicely folded one).

3.1 Melting of Lipids

Melting is a macroscopic phenomena. In general, an entire membrane, or a big piece of it will melt at once. This is called cooperativity. From the description of a single molecule, it is possible to get a picture of what energy is required for a melting transition (in DPPC membranes this is ca 35 kJ /mol). The width of the melting transition (width, as in how many degrees Kelvin from the transition will the two phases still coexist), however can only be found based on the cooperativity.

The simplest way to use cooperativity is to generalize that membranes melts in lumps of, say, 1000 lipids at a time. You can then calculate the melting transition as for one big molecule. The actual size of the lumps will depend on the specific shape of the membrane, what other molecules are nearby, etc, and will de facto have to be found experimentally.

This is still a rough simplification. To be more precise, one would have to do computer simulations.

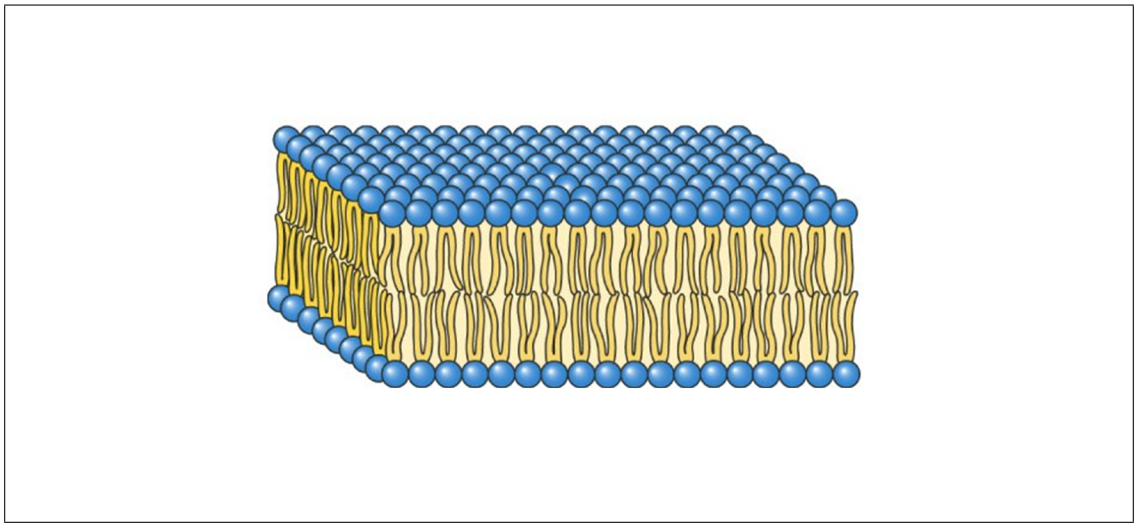


Figure 5: *A membrane. The blue parts are the hydrophilic ends, and the yellow strings the hydrophobic ones. (Picture from amit1b.wordpress.com)*

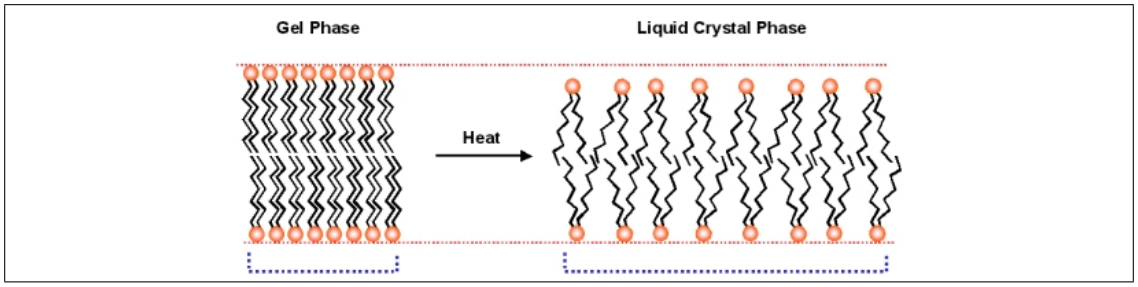


Figure 6: Membranes melting. Notice they keep their shape, but change their volume and area. (Picture from mikeblaber.org)

4 Anesthesia and Thermodynamics

Certain chemicals, ethanol (that is to say normal alcohol) amongst them, has the ability to render humans and other animals unconscious. We call those chemicals anesthetics. When dealing with anesthetics it is normal to rank them after how great a dose is required to render 50% of people taking such a dose unconscious, and then ignore other effects for simplicity. So even though there is a difference in the subjective feeling of for example morphine and alcohol, we will just rank them after how much of each is required to send you to sleep.

The easiest way to test anesthetics is by drugging tadpoles instead of humans. With tadpoles, you can just pour the anesthetic into the water they are swimming in, and assume the concentration in their bodies is the same as the concentration in the water. You can then count how many tadpoles has stopped moving at a

certain concentration. When it's fifty percent, you have the critical concentration, ED_{50}. (So the higher the critical dose, the weaker the anesthetic is).

For reasons that will soon be made clear we will now define the concept of a partition coefficent. The partition coefficent of a chemical, can be found by putting oil and water in the same container and adding the chemical. After some time, the chemical will be distributed between the water and the oil, and the ratio between the concentrations is the partition coefficent

$$P = \frac{[C_{oil}]}{[C_{water}]}$$

For some chemicals the partition coefficent is nearly the same for all oils, for others it vary.

In 1899 and 1901 the two scientists Hans Meyer and Charles Overton each discovered that for most anesthetics, the critical dose is inversely proportional with the the partition coefficent of the chemical[3]. Now, if lipid membranes have the same properties as oils, this would mean that at a critical dose, the concentration of anesthetic molecules in the lipid membranes of the tadpoles are excactly the same, no matter which anesthetic is used.

One explanation of this rule could be: Nerves are made of very long cells. Their walls are off some sort of lipid membranes. It is possible to alter the melting point of a membrane by dissolving small non reactive chemicals with very low melting points in it, as will be described in the next section. This would mean that at critical concentration of anesthetics, the melting point of the nerves will have been lowered by the same amount, independent of anesthetic.

5 Lowering of melting temperature

Only small amounts of certain substances can sometimes have a great influence on the melting point of others. Think of salt and ice. What is required is just that the chemical used is much easier soluble in one phase than in another of the substance it is put into

This short and simplified explanation works in principle in all such situations, not just in lipids.

Let's imagine a lipid with a certain melting temperature at wich it goes from gel to liquid. Then we put in a small amount of a nonreactive chemical with a much, much lower melting temperature, which is only solluble in the liquid phase and not in the gel phase of the lipid. The mix in the liquid phase is an ideal solution(it reacts with fluid lipids excactly like they react with each other).

According to the rules of thermodynamics, we would be able to write the chemical potential of the lipid in the liquid phase as

$$\mu_f = \mu_{f,0} + RTln(x_{l,f})$$

Here μ_f the chemical potential of the lipid in the fluid phase(pr mol), and $x_{l,f}$ is the molar fraction of fluid lipid vs the other chemical. R is the gas constant and T is the temperature.

In these experiments, and also in the human body, the lipids is surrounded by a great amount of water. We assume that the chemical we use has a high solubility in water (which ethanol has). We will therefore assume that the concentration of the chemical in the water is constant, even though a small amount of it goes from the water and into the lipid, when the lipid melts. If we also assume that so little of the chemical is dissolved in the membrane that we can ignore the change in volume of the membrane because of this, then the molar concentration of the chemical in the liquid phase of the lipid depends only on the concentration of the chemical in the surrounding water. The dependence of the concentrations is known as the partition coefficent, which depends on the nature of the chemical.

$$x_{c,f} = P \cdot C_{H_2O} \cdot V_{lipid}$$

Where
$x_{c,f}$ is the molar concentration of the chemical in the fluid phase of the lipid.

P is the partition coefficent of the chemical.

C_{H_2O} is the concentration of the chemical in the water.

V_{lipid} is the molar volume of the lipid (in its fluid phase)

At chemical equilibrium, when there is excactly the same amount of melted and unmelted lipid (which means it is the new melting point)

$$\mu_g = \mu_f$$

Where μ_g is the chemical potential of the lipid in the gel phase.

$$\mu_g = \mu_{f,0} + RTln(x_{l,f})$$

$$x_{l,f} = exp(\frac{\mu_g - \mu_{f,0}}{RT})$$

Now we break the basic difference in chemical potential into its enthalpy and entropy components

$$\mu_{f,0} - \mu_g = \Delta H_0 - T\Delta S_0$$

ΔH_0 and ΔS_0 are the change in enthalpy and entropy for melting one mole of the lipid. Since this should be zero at normal melting temperature

$$\Delta S_0 = \frac{\Delta H_0}{T_{m,0}}$$

Where $T_{m,0}$ is the melting temperarure of the pure lipid.
Putting all this together we get

$$x_{l,f} = exp(-\frac{\Delta H_0}{R}(\frac{1}{T} - \frac{1}{T_{m,0}})$$

We now instead use the fraction of the other chemical. First we know

$$x_{l,f} = 1 - x_{c,f}$$

Using that, we can write

$$ln(1 - x_{c,f}) = -\frac{\Delta H_0}{R}(\frac{1}{T} - \frac{1}{T_{m,0}})$$

Now begins the simplifications.
First we assume that only a very small amount of chemical is dissolved in the lipids ($x_{c,f} << 1$). Then we can use

$$ln(1 - x_{c,f}) \approx -x_{c,f}$$

Using this we get

$$x_{c,f} = \frac{\Delta H_0}{R}(\frac{1}{T} - \frac{1}{T_{m,0}})$$

Then, if we assume that the change in melting temperature is small; $T - T_{m,0} >> T_{m,0}$ (This is fair if we are looking for a change of no more than a few degrees Kelvin, in processes around room temperature, e.g. 300K). Then we can approximate

$$\frac{1}{T} - \frac{1}{T_{m,0}} \approx \frac{T_{m,0} - T}{T_{m,0}^2}$$

Using these two we will finally get the new melting temperature

$$T = T_{m,0} - \frac{RT_{m,0}^2}{\Delta H_0} x_{c,f}$$

Remember that for lipids in a basin of water $x_{c,f} = P \cdot c_{H_2O} \cdot v_{lipid}$. For ethanol, experiments have found $P \approx 0.5$ [1]

A DPPC membrane has a melting change of enthalpy $\Delta H = 35 \frac{kJ}{mole}$ and a melting temperature $T_{m,0} = 41°\text{C} = 314.2K$ and it's molar volume is $V_{lipid} = 0.734 \frac{l}{mol}$ [3].

Using this, we find for ethanol and DPPC membranes

$$\Delta T_m = T_{m,0} - T \approx 14.12 \frac{K \cdot l}{mol} C_{H20}$$

A very simple model indeed

5.1 How good are the assumptions?

Let's discuss this model for the case of ethanol and DPPC lipids.

Now, to make this very simple model we had to make the following assumptions:
1: Ethanol has the same partition coefficent going from water to liquid DPPC as from water to octanol.
2:Ethanol is not at all soluble in gel DPPC, but mixes ideally with liquid DPPC
3:The amount of water to DPPC is so large, that the lipid going from water to DPPC does not reduce the concentration in the water.
4: The molar fraction of ethanol in DPPC is very small, and the shift in melting temperature is no more than a few degrees.

Number 3 and number 4 we can control ourselves, and in this particular experiment they hold. There is a greater problem with 1 and 2.

Ethanol is a tricky molecule, and do actually have different partition coefficients for different fatty substances. It's particular partition coefficent for DPPC has been measured to be 0.22 [4].

As goes for 2, whether ethanol mix ideally with liquid DPPC, I cannot guarantee it. You need experiments like these to find out.

If we use 0.22 for the partition coefficent, we get a shift in melting temperature:

$$\Delta T_m = T_{m,0} - T \approx 6.21 \frac{K \cdot l}{mol} C_{H20}$$

6 Lowering of melting enthalpy

As already mentioned, melting of a membrane requires a certain amount of energy based on the type of lipid. This is just the energy difference between being in the energetically preferred chain configuration, and then being in a perfectly random configuration. But, when the melting temperature is lowered, this required energy is also lowered.

Since at melting temperature, the difference in Gibbs free energy between the two phases still is zero, the new change in enthalpy per mol of lipid can be written as

$$\Delta H = \Delta H_0(1 + \frac{\Delta T}{T_{m,0}})$$

7 Experimental procedure

7.1 Preparation of sample

The lipid needs to be in small unilamellar cells for the experiment. To achieve this, we push the unstructured lipid through a filter with 0.4 μm holes (the correct technical term for pushing something through something else in order to shape it is 'Extrusion'). This is done numerous times, at a temperature higher than the lipids melting point.

The reason for this is: It takes a certain amount for the ethanol to mix from the water to the lipid membrane during the melting. To get a good picture it is necessary to keep this time as small as possible, which is done by making the surface between the membrane and water large.

7.2 ITC

During early runs of the ITC experiment, it became clear that there were no transitions where there should be according to the simple theory. Then, after making titrations over broad regions, the transitions were found to be induced much later(at higher concentrations according to temperature). After enough scans had showed this, I therefore made three precise titrations in these regions, which excactly matched a correlation about three times lower than expected.

These runs was done with 1.7M (10% vol) ethanol, in 100 injections of 2 ul into 1.5 ml of water containing ca 4.75 mM DPPC. After each injection, there was a 250 second pause, to allow the ethanol to mix with the lipids.

7.3 DSC

For some of the DSC experiments, I used excactly the same DPPC batch as in the ITC, for others I mixed a new one. The DSC is able to both heat and cool, and I therefore made both experiments with melting and freezing the DPPC membranes. This gave slightly different results. The freezing temperature was about 0,3 K lower than the melting temperature.

In the machine, I used 0.6 ml of 4.75 mM DPPC. This was first quickly heated from 30°C to 50°C, in half an hour, and then cooled back down in half an hour. After these preliminary runs, there was made two runs from 30°C to 50°C and back again, with a speed of 5°C per hour, during which the data was taken. In all eight different samples was tested.

8 Results

First, I must mention that in the data between the two experiments, there is a difference in absolute temperature by about 0.3 K. The two machines have been calibrated for absolute temperature in slightly different ways. This, however, is not a problem as long as we only look at the *change* in melting temperature, and not at the absolute one.

The ITC raw data is the power the sample cell is heated with during the experiment. To this we first subtract the baseline, that is to say, we only use the change from the effect necessary to keep a fixed temperature, which is induced by the injections. We then integrate the effect over each injection, to get the entire energy added pr injection. Then, since we only are interested in the energy taken by the phase transitions, we subtract the heat of dilution caused by the ethanol. This is done by making titrations with ethanol into water (starting with the same ethanol content as the relevant real experiments), and then subtracting this result.

For the DSC, it is only necessary to subtract the baseline, that is the effect needed to heat the sample when no phase transitions is going on.

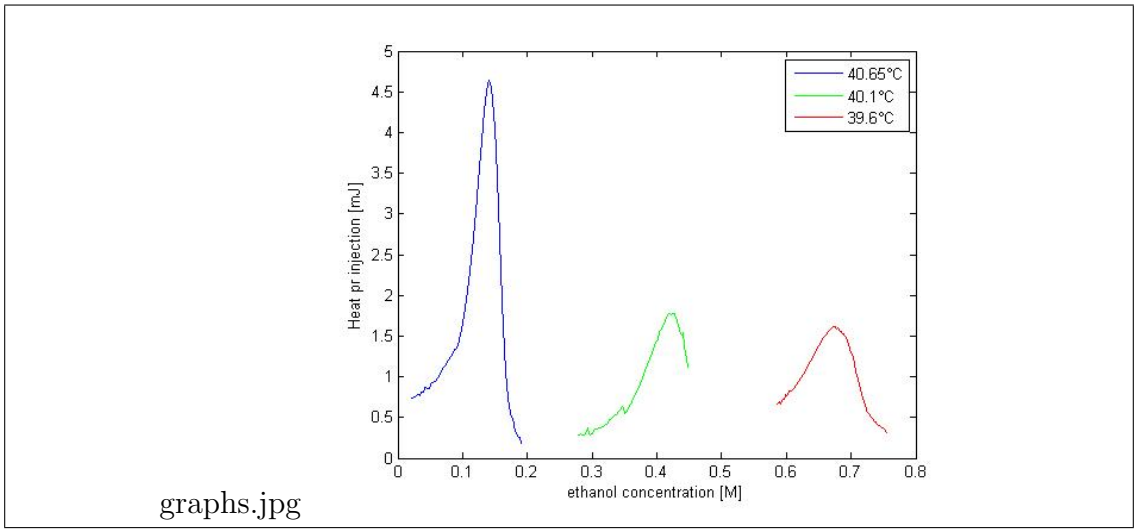
The 3 measurements from the ITC seems to give a simple correlation between melting temperature and ethanol concentration as

$$T = 40.93°C - 1.973\frac{°C \cdot l}{mol}C_{H2O}$$

This is 3 times lower than our theoretical guess.

Making a regression of the eight melting temperatures of the DSC gives

$$T = 41.17°C - 1.388\frac{°C \cdot l}{mol}C_{H2O}$$



graphs.jpg

Figure 7: *Results from the ITC. See figure 2, righthand picture for examples of raw data. Here those single peaks of heat from the injections have been integrated, to yeild the total heat pr injection as a function of total ethanol concentration. The peaks of the melting transitions can be clearly seen, but the transitions are too broad to be completely captured. It is however interesting to notice how excactly the peaks looks like regular melting peaks from heating.*

However, there is one melting point which seems a clear outlier. It is possible this could be a mistake in dosing the ethanol. If we allow ourself to exclude this point we get

$$T = 41.28°C - 1.474\frac{°C \cdot l}{mol}C_{H2O}$$

Fitting to the freezing temperatures on the other hand gives:

$$T = 40.57°C - 0.869\frac{°C \cdot l}{mol}C_{H2O}$$

Here there are two outlier points. The same as for the melting, and the third point which has a very great distance between melting and freezing, compared to the others. If we excluded these points we would get

$$T = 40.95°C - 1.322\frac{°C \cdot l}{mol}C_{H2O}$$

The total melting energy can be found by integrating under the heat capacity peaks. Then from the total amount in the DSC, the melting energy pr mole can be found.

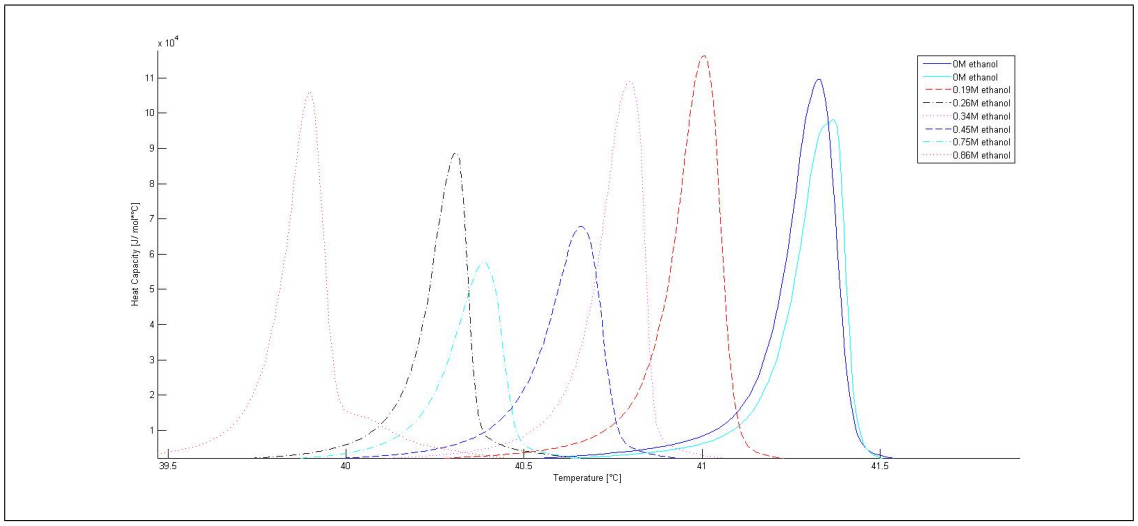


Figure 8: *Results from the DSC. It is clearly visible how the melting temperature is lowered by the ethanol concentration. Notice that here the normal heat capacity of the sample has been subtracted, so leaving only the heat capacity for the melting transition. It can also already here be seen that the concentration of DPPC must have varied between the tests, leading to the differences in shape.*

9 Problems

9.1 Preparation of membranes

All these experiments should be done with 5mM concentration of DPPC. But, during preparation for the experiment, the lipid has to go through a number of processes to get the desired $0.4\mu m$ ball shape, also known as $0.4\mu m$ unilamellar vesicle shape. Now, whenever a small amount of sticky substance has to change containers a lot (which the lipids do during the processes), some will get lost (this is just another example of the second law of thermodynamics in practice). We can therefore only give the upper bound of how high the concentration of lipid is (the concentration we started with), and then say that probably the concentration is somewhat lower, though probably not critically much so. This will off course result in somewhat lower changes in enthalpy during melting, since there is less stuff melting.

To compensate, for lost DPPC, i will use a concentration of 4.75mM +/- 0.25mM during calculations. However, in the end even this might seem to be underestimated, since all the measurements of melting enthalpy is a bit lower than the expected value (around 35kJ/mol).

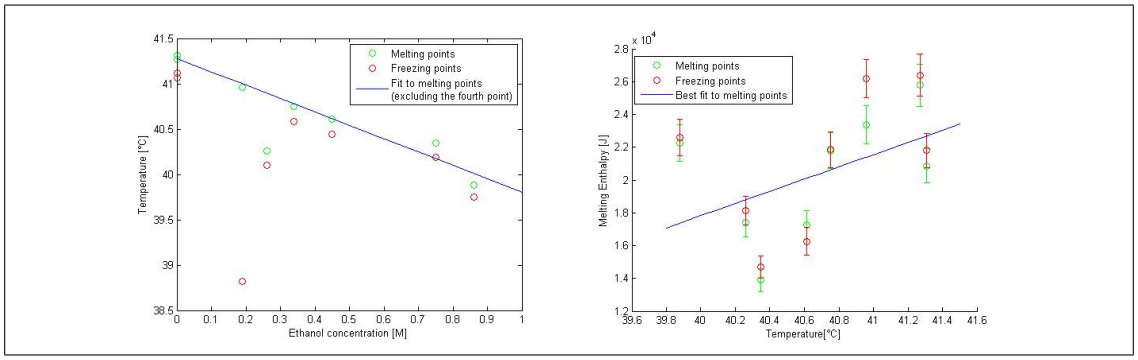


Figure 9: *On the left :Meltingpoint and freezingpoints of DPPC. Notice that there are two different measurements for pure DPPC, lying very close to each other. On the right: Measurements of total enthalpy change during a transition, as a function of melting temperature. The errors are based on the estimated loss of DPPC during preparation. They seem to have been underestimated.*

9.2 ITC

The experiments suffers from some problems. First the temperature of the cell is not kept quite constant. This would off course be impossible, since it is heated and cooled all during the experiment, with the heating varying. De facto, the temperature can swing during an experiment with something like $0,05K$. This is a problem, since this is only one order of magnitude lower than the changes of melting temperature we are observing $(0,5K - 1K)$.

Secondly, ethanol has a very high heat of dilution. Now remember that the sample cell is always heated a bit, and it is the magnitude of this heat that is used as data. Well, if a reaction in the cell produces too much heat, the supplied heat will drop to zero. And since it can't drop under zero (it's a heater not a cooling element), data at such magnitudes will not be precise.

In effect, only very small and not too concentrated injections of ethanol can be made. This is a problem, since the transition is quite broad, so it would have been better to be able to titrate more ethanol during the experiment, thereby getting an altogether broader spectrum. As can be seen from the data, the transition is broader than the measurement, making any calculation of melting enthalpy very imprecise.

Thirdly, there is the possibility of airbubbles in the sample cell. This would mean less total lipid in the cell, which would cause changes in the total observed melting energy, but no shift in melting temperature. Since it anyway isn't possible to measure the melting energy from the ITC data, this is not a great problem-

9.3 DSC

The DSC also had the possibility of bubbles in the cells. This would mean less total melting energy than expected. Also, where the ITC was stirred during the entire experiment, and the transition took place during the entire 8 hour experiment, then in the DSC the transition took place during an interval of no more than 20 minutes, and the cell wasn't stirred. This means that during melting, the dispersion of ethanol in the water can play a role. To be more precise, the experiment could be done slower.

This is one likely explanation for the differences between the transition points when going up and going down in temperature.

10 Discussion

The change in melting temperature was found with the ITC to be -1.97 K*l/mol, and with the ITC to be around -1.47 to -1.32 K*l/mol, depending on what points were used. So both of these are far from our expected value, and they are not excactly the same value either. Within the expected deviations (which seemed to match the measurements in each experiment by itself), this great difference between the two experiments is inexplainable. With more time, calibration experiments between the two equipments might be designed.

These two values would correspond to a partition coefficent between water and DPPC of around 0.07 to 0.05.

11 Conclusion

The partition coefficent of ethanol between DPPC and water is probably lower than 0.22. The measurements of melting enthalpy is too imprecise to be useful.

12 Acknowledgements

Thanks to Thomas Heimburg for being a great advisor, to Henrike Sasse-Middelhoff for helping me with numerous practical problems, and to the powers that be for letting my experiment survive despite electrical irregularities.

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(They are themselves citing *Sarasua et al. 1989*, which I haven't been able to find)