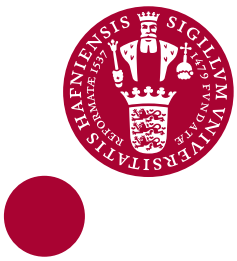

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Published in:
arXiv.org: Physics

Publication date:
2007

Document version
Publisher's PDF, also known as Version of record

Melting of Single Lipid Components in Binary Lipid Mixtures: A Comparison between FTIR Spectroscopy, DSC and Monte Carlo Simulations

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Monte Carlo (MC) Simulations, Differential Scanning Calorimetry (DSC) and Fourier Transform InfraRed (FTIR) spectroscopy were used to study the melting behavior of single lipid components in two-component membranes of 1,2-Dimyristoyl-D54-sn-Glycero-3-Phosphocholine (*DMPC-d54*) and 1,2-Distearoyl-sn-Glycero-3-Phosphocholine (*DSPC*). Microscopic information on the temperature dependent melting of the single lipid species could be investigated using FTIR. The microscopic behavior measured could be well described by the results from the MC simulations. These simulations also allowed to calculate heat capacity profiles as determined with DSC. These ones provide macroscopic information about melting enthalpies and entropy changes which are not accessible with FTIR. Therefore, the MC simulations allowed us to link the two different experimental approaches of FTIR and DSC.

keywords: domain formation; phase separation; FTIR; MC simulation; DSC; melting of single lipid components

Introduction

Biological membranes display a complex lipid composition. The reason for the big variety in lipid species is still not clear. It has been shown for many different bacteria that their lipid synthesis depends on physical parameters such as growth temperature or hydrostatic pressure (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, regard also the citations given therein) . It could be shown that the membranes of bacteria grown at higher temperatures contain more saturated fatty acid chains and chains tend to be longer (1, 2, 3, 4, 5, 6, 7, 8, 11, see also citations therein). Higher hydrostatic pressure induces an increased synthesis of unsaturated lipids (9, 10), which has also been found for biological membranes from several deep-sea fish tissues (12). This adaption has an influence on the physical behavior of a biological membrane such as the melting transition behavior.

Melting transitions from a solid ordered (*so*; in the literature also called *gel*) to a liquid disordered (*ld*; or named as *fluid*) phase are present in artificial and biological membranes (13, 14, 15, 16, 17, 18). In the presence of cholesterol also *liquid ordered* phases develop (19). Considering single lipid membranes one finds that a higher degree of saturation or longer fatty acids result in higher melting temperatures (20). Therefore, one would expect a change of the melting behavior of biological membranes in dependence of growth temperature or hydrostatic pressure and therewith in dependence of lipid synthesis. Indeed, this has been demonstrated for the prominent example of the inner membrane of Escherichia coli (21).

The dependence of lipid synthesis by outer physical parameters such as temperature or hydrostatic pressure was termed "homeoviscous adaption" (22, 23). The underlying idea was that for a proper cell function the membrane needs to provide a certain viscosity. In this context the widely used term "fluidity" was introduced in the literature. It should, however, be noted that this term lacks a clear definition. It was also argued against a role of "fluidity" (24). An alternative role of the adaption of lipid synthesis on outer physical parameters is to ensure a control of the heterogeneity of the lateral membrane structure (25). This idea has obtained special attention

Abbreviations: DSC, differential scanning calorimetry; FTIR, fourier transform infrared spectroscopy; MC simulation, Monte Carlo simulation; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPC-d54, 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine

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within the discussion about "rafts" (26).

Artificial lipid membranes consisting of one, two or three lipid components are suitable systems to study the general physical behavior of lipid phase transitions in more complex lipid systems . In the phase coexistence regime domains with a size ranging from a few nanometers to several micrometers form in model systems and can be observed by a large variety of experimental techniques, such as Fluorescence Resonance Energy Transfer, Atomic Force Microscopy and Confocal Fluorescence Microscopy (27, 28, 29, 30, 31).

Already since the early 1970s the idea that domain formation processes can influence protein activity and function was expressed (25, 32, 33). In several publications the existence of domains have been shown to provide a mechanism to control biochemical reaction cascades (34, 35, 36, 37, 38). Evidence that lipid phase transitions and domain coexistence trigger enzyme activity has been found for various systems. For example, the enzyme phospholipase A_2 which hydrolyzes the *sn-2* bond of phospholipids, producing a free fatty acid and a lysolipid, is strongest in activity when the lipid membrane is in the lipid phase transition regime (39, 40, 41). The activity of the special class of phospholipase A_2 type II A has been demonstrated to be related to an enrichment of anionic lipids into *ld* domains (42). In this case, a different partition behavior of the anionic lipids into *ld* and *lo* domains led to a local concentration higher than the threshold value for enzyme activity. Changes in the opening times and probabilities of calcium channels reconstituted in POPE:POPC membrane of various ratios have been interpreted to be due to the coexistence of solid ordered and liquid disordered phases (43). A further example is the protein kinase C (PKC) which modifies proteins by chemically adding phosphate groups. It has been discussed that lateral heterogeneities of the lipid membrane control the activation of this enzyme (44, 45). In the latter study it has also been pointed out that domains enriched in dioleoylglycerol lipids play a crucial role, but also a linear relationship between PKC activity and phosphatidylserine content has been found (46). Further communications can be retrieved where a connection between enzyme activity and the presence of specific lipids and structure are made. This is e.g. also the case for the calcium ATPase (47).

The publications cited above are only a small fraction of many in that respect, but they already show that it is important not only to study domain formation, but also to investigate the fraction of the single lipid species in lipid membranes showing lateral inhomogeneity due to phase separation. The single lipid species will occur in different amounts in the different domains that form in a phase transition regime (48). The work presented here concentrates on this for the case of a binary lipid mixture composed of *DMPC* and *DSPC*. These two phospholipids display melting transition events with transition midpoint temperatures of $23.6°$C or $54.4°$C, respectively (20). In our study we used a deuterated *DMPC* derivate whose phase transition midpoint temperature lies at around $19 - 20°C$ (49, 50).

For the description of phase separation in lipid membranes various theoretical approaches have been applied. This includes ideal and regular solution theory, mean field, but also numerical approaches such as Monte Carlo (MC) simulations (48, 51, 52, 53, 54). Ideal and regular solution theories allow the understanding of phase diagrams. In the ideal solution theory complete miscibility in the solid ordered and liquid disordered phase is assumed. The regular solution theory takes a step further and regards a non-ideal mixing in the solid ordered phase. These considerations allow the construction of phase diagrams. Phase diagrams are used to map the phase behavior of lipid membranes in dependence of e.g. temperature and lipid composition. The theoretically derived lever rule provides the possibility to determine the ratio of disordered and ordered lipids in dependence of temperature and lipid composition of binary lipid mixtures and can also be applied to experimentally measured phase diagrams (48). Ideal or regular solution theory are, however, based on simplifying assumptions. This does not only include an ideal mixing in the liquid phase, but also the assumption of phase separation under all conditions in the melting regime. This is, however, not the case (55). Further, fluctuations which are enhanced in the melting transition regime are present (56, 57). This is where MC simulations analyzing simple models of lipid chain melting enter. They allow the consideration of interactions between different lipid species and varying chain state. The formation of lipid domains and phases and the consideration of fluctuations is a direct consequence of the thermodynamics of these systems which is included in these models. Therefore, these simulations can describe lipid membrane properties in the vicinity of lipid phase transitions, such as changes in membrane permeability (58), fluctuations in enthalpy (57) and diffusion processes (59, 60) well.

In this study we determined the amount of the single lipid species in the different domains of binary *DMPC* and *DSPC* mixtures by means of MC simulations and compared the results obtained to experimental data measured by Fourier Transform InfraRed spectroscopy (FTIR).

Among other techniques FTIR is well suited to investigate the melting behavior of lipid membranes. In these studies the temperature dependence of various vibrational modes due to structural changes provide a versatile tool in following phase transitions (61). Articles have been published on experiments in which FTIR was used to measure nanoscale domain size (62) and chain order parameters (63).

In our study the hydrogen atoms of *DMPC* were replaced by deuterium atoms. Due to this the melting of the *DMPC* lipid chains could be separated from the *DSPC* lipid chain melting, as the CH_2 and CD_2 symmetric and asymmetric stretch vibrations differ in frequency due to the heavier deuterium atoms. This allowed us to compare the melting of single components with MC simulation results which contain information of the disordered chain ratio of single lipid components.

MC simulations are able to describe experimental heat capacity profiles well. Therefore, they provide a means to couple the two different experimental approaches.

The experimental absorption spectra were evaluated using two methods. One of the methods probed the temperature dependence of the position of the absorption maxima of the antisymmetric CD_2 and symmetric CH_2 stretch vibrations. The other one evaluated peak area changes of difference spectra (*Difference Spectra method*). Simulation results on the fraction of disordered chains of the single lipid species agreed well with the data found using the latter method. FTIR allows to distinguish the melting of the single lipid species and it probes the microscopic behavior. DSC probes the bulk behavior and macroscopic observables. The Monte Carlo simulations were used as an approach to combine the informations of the two experimental techniques. DSC, MC and FTIR are a potent combination to investigate the melting behavior of lipid membranes.

Materials and Methods

Sample Preparation:

The lipids 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine (*DMPC-d54*) and 1,2-distearoyl-sn-glycero-3-phosphocholine (*DSPC*) were purchased from Avanti Polar Lipids (Alabaster, USA). Samples were prepared from lipid stock solution in chloroform. These were prepared in chloroform from lipid powder as delivered from the vendor without further purification. To prepare lipid samples in buffer, chloroform from the desired amount of lipid stock solution mixture was evaporated under a stream of nitrogen and the sample was kept under vacuum for several hours afterwards. After this procedure the dried lipid film was hydrated with preheated ultrapure water at a temperature of 60°C, which is higher than the melting temperature of the higher melting lipid component *DSPC* (54.4° C, (20)) and kept stirring at this temperature for about 30 minutes. In calorimetric experiments a lipid concentration of $10\ mM$ was used. The Fourier Transform Infrared spectroscopy measurements required concentrations of $65\ mM$. In order to facilitate lipid membrane hydration under these high concentrations the samples were subjected to several freeze-thaw cycles.

DSC experiments:

Differential Scanning Calorimetry (DSC) experiments were performed on a VP-DSC (MicroCal, Northampton, MA, USA) with a scan rate of 15°C/h (DMPC-d54:DSPC $50:50$) or of 5°C/h (DMPC-d54) per hour in upscan direction. The samples were equilibrated at the starting temperature for 30 minutes.

Fourier Transform InfraRed spectroscopy experiments:

Fourier Transform InfraRed (FTIR) spectroscopy experiments were performed on a Vertex70 (Bruker Optics, Bremen, Germany) equipped with a mercury-cadmium-telluride (MCT) detector. The spectrometer was flushed with nitrogen starting about 30 minutes before the experiments. Temperature control was done by a self built sample holder connected to a water bath (DC30-K20, ThermoHaake, Karlsruhe, Germany). The scan rate for the temperature ramp was set to 20°C/h. The temperature in the sample corresponding to each temperature point set with the water bath was appointed in a reference scan. Background spectra were acquired at each temperature point after lifting the sample compartment out of the beam path. We were interested in the IR absorption due to the symmetric and asymmetric stretch vibrational modes of the CH_2 and CD_2 groups of the lipid fatty acid chains. Example absorption maxima of the CH_2 symmetric and asymmetric stretch vibrations at different temperatures are displayed in the left panel of fig. 2.

Two methods to analyze the FTIR spectroscopy data were used. In a first approach FTIR spectroscopy data was processed with a self-written plugin for the IGOR software package (WaveMetrics, Lake Oswego, OR, USA). The absorption peaks were fitted with a polynomial function. Then the peak position was determined by calculating the root of the fit.

For the second protocol the peak area of the CH_2 or respectively of the CD_2 stretch vibrations was normalized to a value of $1 \times cm^{-1}$. A reference spectrum was chosen which was taken at a temperature below the melting transition regime. This spectrum was subtracted from all other spectra which were recorded at different temperatures (see the right panel of fig. 2). Then the peak area located at the positive axis of ordinates was calculated and plotted as a function of temperature. There was no difference in surveying both, symmetric and antisymmetric modes or the single ones. Further, we shall call this method the *Difference Spectra method*.

Model:

We have already applied an Ising-like model to the study of diffusion (59), fluctuation (57) and relaxation properties (64) of *DMPC:DSPC* lipid mixtures. In this paper we used this model to describe FTIR measurements on mixtures of *DMPC-d54* and *DSPC* and linked these with DSC measurements.

The model has been presented in detail elsewhere (54, 59). In short, the model is based on the assumption that the lipid chain states can be described by assigning either an ordered or disordered state to the fatty acid lipid chains. Lipid chains are arranged on a triangular lattice and only nearest neighbor interactions are included. Lipid chains might change the degree of order or their positions during the simulation. The model is evaluated with Monte Carlo (MC) simulations in which the free energy difference of an old and a trial configuration is sufficient for decision making of the acceptance of a new configuration. The free energy of the system can be divided into a configuration dependent and an independent part. The configuration dependent contribution equals (54):

$$\begin{aligned}\Delta G(\mathbf{S}) &= N_1^d(\Delta H_1 - T\Delta S_1) + N_2^d(\Delta H_2 - \Delta S_2) \\ &+ N_{11}^{od}\omega_{11}^{od} + N_{12}^{oo}\omega_{12}^{oo} + N_{12}^{od}\omega_{12}^{od} \\ &+ N_{12}^{dd}\omega_{12}^{dd} + N_{12}^{do}\omega_{12}^{do} + N_{22}^{od}\omega_{22}^{od},\end{aligned} \quad (1)$$

where ΔH_i and ΔS_i are the enthalpy and respective entropy differences of the disordered to the ordered state of *DMPC* ($i = 1$) and *DSPC* ($i = 2$) lipid chains. ω_{ij}^{mn} are interaction parameters of unlike nearest neighbors and N_{ij}^{mn} the corresponding number of the unlike nearest neighbor contacts with $(i \neq j)$ and $(m \neq n)$. m and n denote the state of a chain which is either ordered (o) or disordered (d). Enthalpy, entropy and nearest neighbor interaction parameters can be obtained from experimentally determined heat capacity profiles. Previously, we have determined the parameters needed for the *DMPC:DSPC* system (59). In this paper, however, FTIR experiments were performed with *DMPC-d54* and *DSPC* mixtures. In the following it was assumed that the unlike species interaction parameters did not change from the system *DMPC:DSPC* to *DMPC-d54:DSPC*. The heat capacity profile of the pure *DMPC-d54* was measured to obtain its enthalpy and entropy changes during the melting transition and the cooperativity parameter ω_{11}^{od} (*data not shown*). The remaining parameters were taken from a recent publication (59). All values are listed in table 1.

$T_{m,1}$ = 19.7	°C	ω_{11}^{od} = 1267	J/mol
$T_{m,2}$ = 54.8	°C	ω_{22}^{od} = 1474	J/mol
ΔH_1 = 14500	J/mol	ω_{12}^{oo} = 607	J/mol
ΔS_1 = 49.5	J/(mol °C)	ω_{12}^{dd} = 251	J/mol
ΔH_2 = 25370	J/mol	ω_{12}^{od} = 1548	J/mol
ΔS_2 = 77.36	J/(mol °C)	ω_{12}^{do} = 1716	J/mol

Table 1: Parameter values used in the Monte Carlo simulations. They were determined from experimentally determined heat capacity profiles (59). All numbers are given per lipid chain. The indices *o* and *d* stand for ordered and disordered, respectively. *1* and *2* index *DMPC-d54* and *DSPC*.

The simulations were done on matrix sizes 60×60 (*DMPC-d54:DSPC* 100:0, 70:30, 60:40, 50:50, 40:60, 30:70), 100×100 (90:10, 80:20, 20:80, 10:90) and 350×350 (*DSPC*) (54). The system was equilibrated for 5000 or around the heat capacity maxima for 10000 MC cycles. Simulations were conducted over 50000 or 100000 MC cycles, respectively.

Results

In this paper we discuss melting processes in binary lipid mixtures. Thereby, we focus on the melting of the two single components independently. Experimental FTIR and DSC measurements were accompanied by numerical simulations and an analysis of the corresponding phase diagram.

MC simulations were used to evaluate a simple model based on the assumption of two lipid chain states, the consideration of nearest neighbor interactions only and the application of a hexagonal lattice. This model has already been shown to describe melting processes in *DMPC:DSPC* mixtures successfully (54, 57, 59). The evaluation needs the determination of ten parameters. These are twice enthalpy and entropy differences between the two disordered and ordered chain states and six unlike nearest neighbor interaction parameters. The enthalpy and entropy changes can be deduced easily from heat capacity measurements of the single one component systems. Two interaction parameters describe the cooperativity, meaning the width of these transitions (65, 66). The four parameters remaining need to be deduced from comparisons with c_p-profiles obtained for different lipid compositions. Previously, we have determined the parameters for a *DMPC:DSPC* system (59). In this study, however, we exchanged the *DMPC* lipids with deuterated *DMPC (DMPC-d54)* which gives heat capacity profiles which are broadened and shifted by $-3.9°C$ to lower temperatures in comparison to the nondeuterated *DMPC* (*data not shown* and see (49, 50)). This means that we had to determine the enthalpy and entropy changes and the cooperativity parameter of pure *DMPC-d54*. In principle using this model one needs to reestablish all parameters for any binary lipid system. In this work, however, we assumed that the unlike nearest neighbor interaction parameters do not change by the replacing of *DMPC* with its deuterated analog and we adopted them from previous simulations of the nondeuterated *DMPC:DSPC* system (59). To validate this, DSC measurements on an equimolar *DMPC:DSPC* mixture were performed and they were compared to results simulated. We found that the values calculated describe the melting profile measured well (see fig. 1). Therefore, we were convinced that it was feasible to further use the model with the interaction parameters given in table 1.

The further scope of this paper was to compare the temperature dependence of the simulated disordered chain ratios of *DMPC-d54* and *DSPC* lipids with the experimental analysis of the melting processes using FTIR spectroscopy. This indirectly allowed us to compare the experimental results obtained with DSC and FTIR.

In FTIR spectroscopy the absorption of infrared light due to vibrational degrees of freedom in the sample is studied. In lipid suspensions different vibrational modes of the lipids contribute to the absorption spectrum. Prominent examples used in our study are CH_2 or CD_2 (in the case of the deuterated *DMPC* lipids) stretch vibrations of the fatty acid lipid chains. The shape of the absorption band is temperature dependent as can be seen in the left panel of fig. 2. Example spectra of the symmetric and asymmetric CH_2 vibrational spectra are given for different temperatures of a mixture of *DMPC-d54:DSPC 40:60*. Deuterium atoms are heavier than hydrogen atoms. This leads to a shift of the CD_2 stretch vibrations to lower wavenumbers and allows to distinguish the

two different lipid species in a binary lipid mixture.

Comparison of FTIR Results and Monte Carlo Simulations

In an investigation of structural changes in cytochrome c Heimburg and Marsh (67) focused on temperature dependent changes of difference spectra. This was motivated by the idea that the corresponding amide I band is a convolution of a variety of different bands. The temperature dependence of each of the single bands differs and a complex temperature dependent behavior is present. Therefore, we decided not only to investigate the temperature dependence of the absorption maxima as done in the literature previously (28, 68, 69), but also to use a *Difference Spectra method* too. Further we studied the peak area of these difference spectra as a function of temperature.

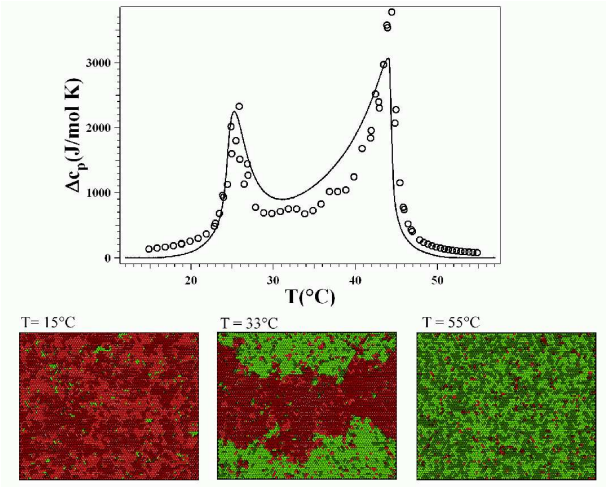

Figure 1: *Enthalpy and entropy changes and cooperativity parameters of the single DMPC-d54 and DSPC lipid membranes were determined from heat capacity profiles. The remaining four unlike species nearest neighbor interaction parameters of DMPC-d54:DSPC mixtures were assumed to equal the ones of a DMPC:DSPC system (59). Simulating an equimolar mixture of DMPC-d54 and DSPC gives a melting profile (markers) which describes well the measured heat capacity profile (solid curve). Below three representative snapshots from simulations of the same mixture with a matrix size of* 80×80 *lipid chains are shown. The red and green colors represent ordered or disordered lipid chains, respectively. The lighter colors correspond to the DMPC-d54 lipid chains and the darker ones to the DSPC lipid chains. The snapshots were taken at three different temperatures. At a temperature of* $15\ ^\circ$ *C the membrane is in the solid ordered phase while at a temperature of* $55\ ^\circ$ *C it is in the liquid disordered phase. In both cases lipids do not mix ideally, but lateral hetereogeneities are present. Two macroscopic phases are present at a temperature of* $33\ ^\circ$ *C.*

In the left panel of fig. 2 example spectra of the CH_2 stretch vibrations are given as a function of temperature of a *DMPC-d54:DSPC 40:60* mixture. Normalizing the area of the spectrum to $1 \times cm^{-1}$ and defining a reference spectrum which lies at a temperature below the melting transition regime, difference spectra were calculated. Representative examples are displayed in the right panel of fig. 2. To follow the melting transition the change in difference spectra peak area of the CH_2 or the CD_2 symmetric, asymmetric or both vibrational modes can be monitored. Our experiments showed that there is no difference between these three approaches (*data not shown*).

The temperature dependent evolution of the difference spectra peak areas monitored are given in fig. 3 as solid curves for four different *DMPC-d54:DSPC* mixtures: (A) *70:30*, (B) *50:50*, (C) *40:60* and (D) *30:70*. The panels on the left side depict the results related to the deuterated *DMPC-d54* lipids and the right ones give the data belonging to the *DSPC* lipids. The data obtained is also compared to simulation results of the ratio of disordered lipid chains for each of the single species. In fig. 3 these are given as open circles (*DMPC-d54*) or squares (*DSPC*). In all instances we found an increase of the area with higher temperatures. In all cases a good agreement between the simulated and measured values is present. The deviations of the data simulated from the measured ones are smaller in the cases of the *DMPC-d54* lipids. This might be due to the fact that the determination of the model parameters needed is imperfect. More extended and time-consuming simulations might improve the data, too.

As already mentioned above, previously melting processes of lipid membranes investigated by FTIR were analyzed monitoring the temperature dependence of the position of the absorption maximum of either the symmetric or asymmetric stretch vibrations of CH_2 and CD_2. We analyzed the temperature dependence of the absorption maxima for several *DMPC-d54:DSPC* mixtures as earlier done by Leidy et al. (28), too. The results obtained are given by the dashed curves in fig. 3.

We confirmed what has been reported by Leidy et al. (28) previously. There are instances when the positions of the maxima of the symmetric stretch vibrations of the *DSPC* lipids do not increase with higher temperatures, but that for certain lipid mixture fractions they decrease, approach a minimum and then they start to become larger again. The minimum wavenumber is in all cases lower than the wavenumbers determined at lower temperatures. This behavior is in disagreement with the evolution of the difference spectra peak area and the MC simulation results.

It is well known that macroscopic, but also local fluctuations of various membrane properties are enhanced in the vicinity of lipid membrane melting transitions (56, 57). The determi-

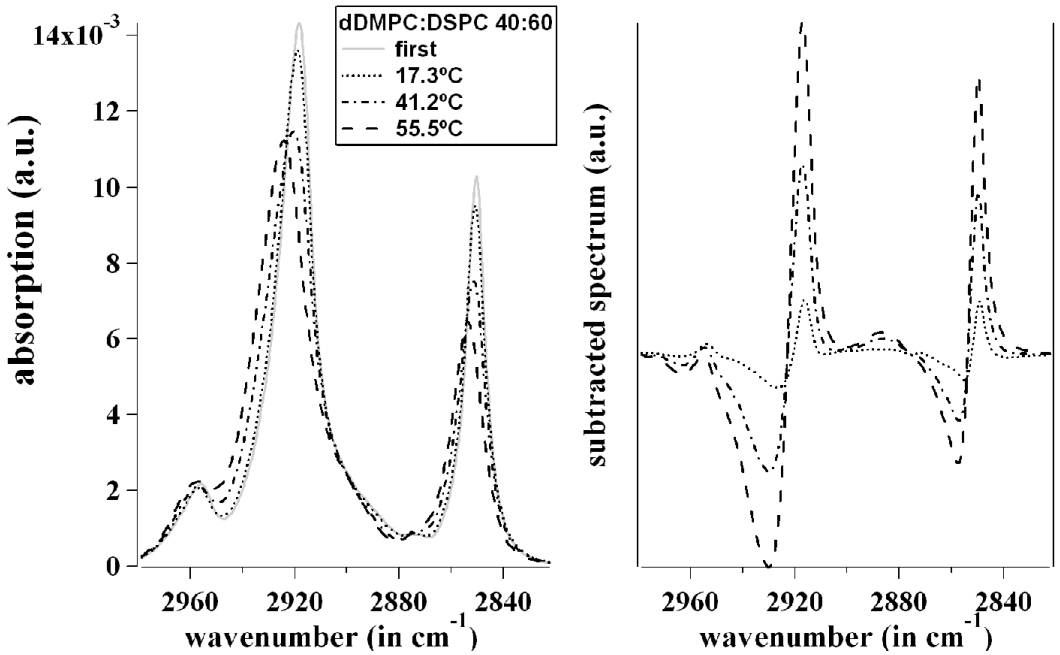

Figure 2: *The IR absorption spectrum contains different absorption maxima originating from varying vibrations. In the left panel the asymmetric and the symmetric CH_2 stretch vibrations as measured in a sample of DMPC-d54:DSPC 40:60 at different temperatures are displayed. The absorption maxima of these are temperature dependent and lie around $2852\ cm^{-1}$ (symmetric) and at $2925\ cm^{-1}$ (asymmetric). The asymmetric and symmetric CD_2 stretch vibrations are shifted to lower wavenumbers due to the heavier deuterium atoms (data not shown). In previous studies from other labs the melting processes were investigated following the temperature dependence of the absorption maxima. In this study, however, the melting process was also studied by defining a reference spectra and subtracting this one from all other spectra where in each cases the area was normalized to $1 \times cm^{-1}$ (Difference Spectra method). Example results of the difference spectra of the left panel are displayed in the right panel. As a measure of the progress of the melting process the area of one of the peaks was taken.*

nation of the derivatives of the data of the curves from fig. 3 as done by Leidy and collaborators(28) provides a measure of the strength of fluctuations with respect to the single lipid components. In fig. 4 these derivatives are shown for the data simulated. The higher the value of the derivative the stronger the fluctuations. *DMPC-d54* melts at lower temperatures than *DSPC*. This is why the corresponding derivatives in the left panels of fig. 4 have maxima at lower temperatures. This temperature, however, depends on the molar fraction of *DSPC* lipids and is higher with an increased *DSPC* lipid fraction. This is similar for the *DSPC* lipids only with the difference that the presence of *DMPC-d54* lowers the temperatures at which fluctuations of the *DSPC* lipid chains are the strongest. In this context it should be noted that the component which is in the minority always displays two maxima. One of the maxima is a local maximum at which fluctuations are enhanced, but they are lower than at the global maximum. The local maximum is found at a temperature at which the other component displays its strongest fluctuations. At an equimolar ratio of *DMPC-d54:DSPC* this is true for both components. The melting transition of the single lipid components happens over a broad temperature regime.

The Phase Diagram

Phase diagrams are generally constructed to easily access the phase separation behavior of complex lipid mixtures (48). In our case a phase diagram depending on lipid composition and temperature was constructed. A phenomenological method to construct these is in aligning tangents on the lower and upper temperature limits of heat capacity profiles. This results in the construction of a solidus and a liquidus line as indicated by open squares and the grey curves in fig. 5. If the lipid composition is known, the physical state of the membrane can be determined. The data obtained from the lower temperature limit defines the solidus line, whereas the one from the upper temperature limit gives the liquidus line. Below the solidus line the lipid membrane is in the *solid ordered* phase, while above the liquidus line it is in the *liquid disordered* phase. Therewith, the liquidus and the solidus lines

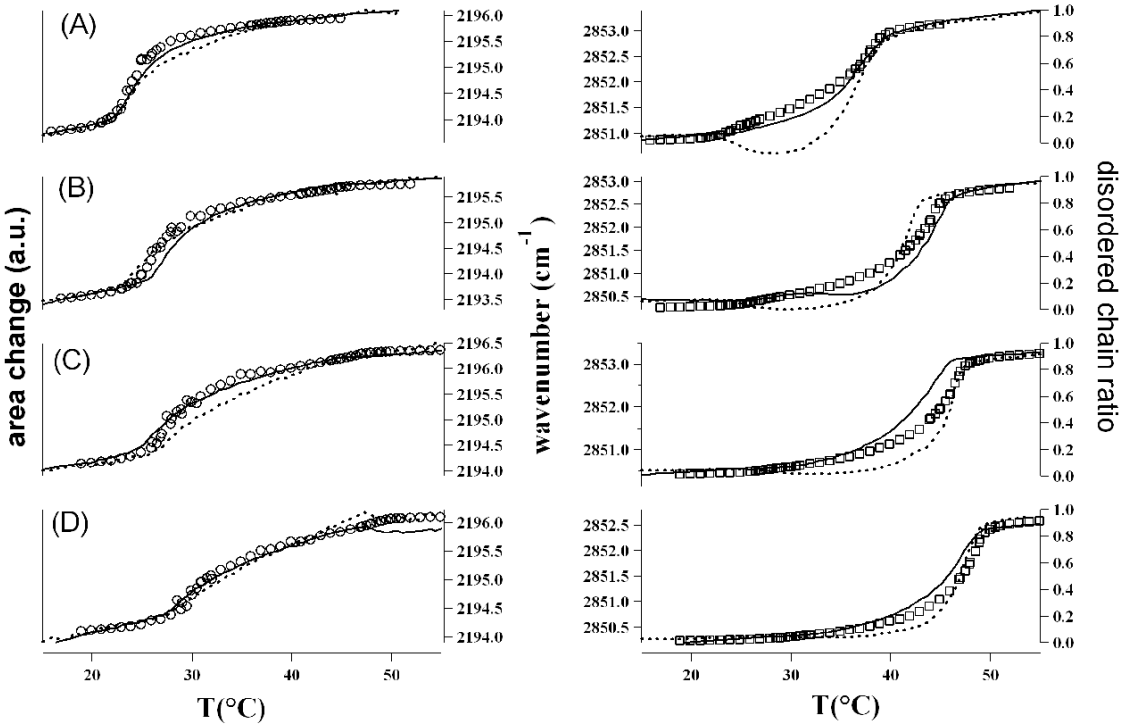

Figure 3: *The FTIR results are compared with the Monte Carlo simulations. The data on four different DMPC-d54:DSPC ratios 70:30 (A), 50:50 (B), 40:60 (C) and 30:70 (D) are displayed. In each panel the disordered chain ratios obtained from the simulations (open circles (DMPC-d54) and open squares (DSPC)) are compared with the changes of the peak area (solid curves) and the evolution of the position of the absorption maximum (dashed curves). In the latter case the antisymmetric CD_2 (DMPC-d54) and the symmetric CH_2 stretch vibrations were followed. The panels on the left side depict the melting process of the deuterated DMPC-d54 lipids and the right ones the DSPC lipid melting. The Monte Carlo simulations allow to combine both the FTIR and the calorimetric results. They provide a mean to relate the macroscopic with the microscopic behavior of the binary lipid membrane melting.*

define the phase coexistence regime too. The three snapshots in fig. 1 provide a visual impression of this. At temperatures of 15° C and 55° C the lipid membrane is in either the *solid ordered* or *liquid disordered* phase, respectively. It should, however, be mentioned that the mixing of the two lipids is in neither of the two cases ideal. The single lipid species cluster. At a temperature of 33° C the two phases coexist and a macroscopic phase separation is present. In an earlier publication we could show that discussing domain formation processes in lipid membranes one needs to distinguish microscopic and macroscopic phase separation and local fluctuations in chain state enter the physical picture. These fluctuations are enhanced at domain or phase boundaries. Small domains are also subject to strong fluctuations (57). Analyzing phase diagrams one should note that these physical effects are in general not considered in its interpretation. Further, it has been pointed out that the construction of a phase diagram is strictly speaking only valid if the transition is of first-order (70). The transition of binary lipid mixtures of *DMPC* and *DSPC* has been claimed to be of second order and therefore the interpretation of the phase diagram has to be taken with care (55).

With the determination of the derivatives of the curves in fig. 3 we were able to deduce the temperatures at which the fluctuations of the single components were enhanced. The lower temperature melting component, in this case *DMPC-d54*, has its maximum at lower temperatures than the one which melts at higher temperatures (*DSPC*). These temperatures, however, are higher or respectively lower than the melting temperatures of the pure single components. The values are given in fig. 5. Open circles refer to the values originating from the simulations while the crosses come from the experiments. The temperature points determined using the two different methods to analyze the FTIR absorption spectra are the same (*data not shown*). The experimental and the simulated data agree well.

The simulations further allowed us to compare the temperatures at which the heat capacity curves are in a maximum and

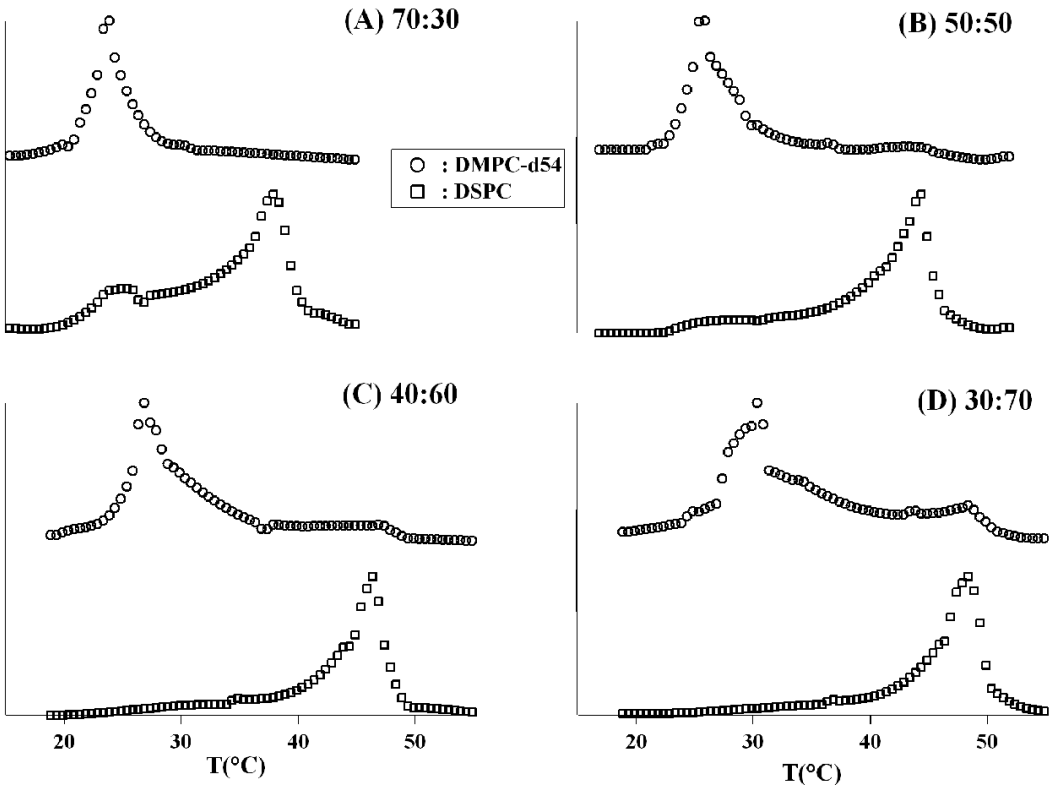

Figure 4: *The derivatives of the curves of the fraction of disordered DMPC-d54 and DSPC lipid chains as shown in fig. 3 are displayed in this figure. In detail these are the mixtures 70:30 (A), 50:50 (B), 40:60 (C) and 30:70 (D). The derivatives belonging to DMPC-d54 are indicated by open circles, while the ones of DSPC by open squares. A maximum in the derivative means that at this temperature fluctuations are enhanced.*

the temperatures at which the derivatives of the disordered chain ratios display their maxima. We found that these temperatures equal each other (*data not shown*). In some cases only one heat capacity maximum is resolvable. The analysis of the single components, however, allows to distinguish the maxima of the melting of single components in all instances. The maxima of the component which is in a majority then agrees with the maximum of the heat capacity curve.

In total, we showed a combined FTIR and MC simulation study. FTIR absorption spectra were analyzed with two methods. One followed the temperature dependence of the absorption maxima of the symmetric or asymmetric CH_2 or CD_2 stretch vibrations. The other studied temperature dependent changes of the peak area of the difference spectra. Monitoring the positions of band maxima leads to incorrect results, but the simulated data describe well the curve shapes obtained with the *Difference Spectra method*. Our simulations describe well the evolution of the area changes. Melting of single components cannot be investigated in DSC. DSC alone only shows the bulk behavior. In FTIR spectroscopy it is possible to separate the lipid species individually. Therefore, using MC simulations we were able to link the two experimental approaches.

Discussion

In this paper we presented a combined numerical and experimental study exploring melting processes in binary lipid mixtures. In detail, we focused on the melting of the single lipid components *DMPC-d54* and *DSPC* independently. Monte Carlo simulations linked FTIR and DSC measurements to each other and therewith MC simulations proved another time to be a useful and powerful tool in exploring melting transitions in lipid membranes.

FTIR, DSC and MC Simulations

FTIR allowed us to measure the absorption spectra of lipid suspensions consisting of MLVs with varying *DMPC-d54* and *DSPC* ratios in dependence of temperature. In particular we probed the CH_2 and CD_2 vibrational modes of the lipid fatty acid chains. The deuterium is heavier than the hydrogen atom which is why the symmetric and asymmetric bands of the CH_2 and CD_2 stretch vibrations display absorptions at dif-

ferent wavenumbers. This made it possible to distinguish the *DMPC-d54* and *DSPC* lipids.

We applied two different methods to analyze the absorption spectra measured. One was based on monitoring the temperature dependence of absorption maxima corresponding to the CH_2 and CD_2 stretch vibrations, respectively. The other one was built on following changes in the peak area of difference spectra (*Difference Spectra method*). We compared the corresponding experimental results to the temperature dependent ratio of disordered chains of the single lipid species using MC simulations of a simple model (see fig. 3).

These numerical simulations were based on a Doniach model of the lipid chain melting process assuming one ordered and one disordered lipid chain state. Nearest neighbor interactions only were considered and the lipid chains were arranged on a triangular lattice. Even though it is a minimalistic model which neglects that lipid chains may reflect different degrees of disorder and the non-consideration of a loss of lattice order during the melting process the calculated fraction of disordered lipid chains of the single lipid species describes the experimental data obtained with the *Difference Spectra method* well.

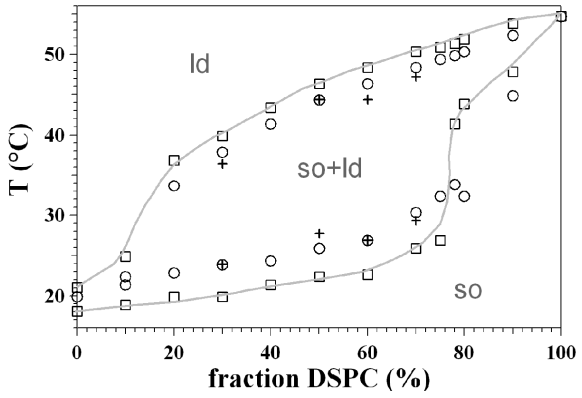

Figure 5: *Phase diagrams allow to obtain an understanding of the phase behavior of lipid mixtures in dependence of lipid ratios, temperature, pH or other outer parameters. In this study binary lipid mixtures were used to study melting transitions in dependence of temperature. Therefore, a phase diagram with the fraction of DSPC lipids as the abscissa and temperature as the ordinate was constructed. The open squares and the grey lines represent the lower and upper temperature limits of the melting process as determined from heat capacity profiles simulated using the tangent method. Further the peak positions of the derivatives of the disordered chain fractions simulated (open circles) and of the experimentally determined area changes (crosses) were analyzed. The simulated peaks determined from the derivatives agree with the maxima of the heat capacity profiles (data not shown)*

Previously this model has been shown to be suited to describe heat capacity profiles as measured with DSC (54, 57, 59). Ten unique parameters are needed which were obtained from experimental heat capacity profiles. Six of them follow directly from the heat capacity curves of the one component systems. These are twice entropy and enthalpy changes and the corresponding cooperativity parameters. The missing four parameters have to be found by fitting the heat capacity profiles of the binary mixtures. They must be and they are the same for all of the possible lipid ratios. Due to the errors in the c_p-profile measurements the parameter determination is subject to deviations from the ideal set of parameters. In our case we have taken the remaining four parameters from the *DMPC:DSPC* system (59). This proved to be valid, but small deviations due to this assumption cannot be excluded, too.

Heat capacity profiles contain macroscopic information on the melting behavior such as changes in enthalpy and entropy. However, DSC does not allow to determine the microscopic behavior of the melting process. Additionally, it does not allow to investigate the melting of the single components. It is only suited to measure the bulk melting behavior. FTIR, however, provides a mean of studying the melting of single lipid species and observes the microscopic details, but lacks the possibility to probe macroscopic properties. The model underlying the MC simulations describes heat capacity profiles and predicts the findings from FTIR well. Therefore, the MC simulations provide a mean to link the information of the two experimental techniques.

The Melting Process

The melting process of single components is adequately described using the numerical simulations. From the experimental and the simulated results and their corresponding derivatives it becomes clear that the lower temperature melting component (*DMPC-d54*) melts also in the binary mixture at a lower temperature than do the *DSPC* lipids (fig. 4). Still, both lipid species melt over the whole temperature regime and not only at distinct temperatures at which fluctuations assigned to the single lipid components are enhanced. In general, the component which is in the minority shows two maxima. At one of these temperatures the strength of the fluctuations of the component which is in the majority is the highest. This effect underlines the cooperativity of the melting process. The melting of one lipid increases the probability of the melting of another lipid.

The temperatures at which the fluctuations of the two components is strongest as obtained from the simulations and the experiments, agree well (fig. 5). Further, the numerical simulations allowed to calculate the heat capacity values and the disordered chain fractions simultaneously. Therefore, we were able to compare the temperatures at which the heat ca-

pacity is in its maximum with the temperatures determined from the derivatives of the disordered chain fractions. These temperatures agree with each other. In fig. 1 the heat capacity profile of the equimolar mixture displays two maxima. The maximum at lower temperature corresponds to the enhancement in fluctuations of the *DMPC-d54* lipids and the one at higher temperatures to the one of the *DSPC* lipids. However, there are instances at which the heat capacity profile displays only one maximum even though one can determine two temperatures using the evolution of the single lipid disordered chain ratios. This is because DSC describes the bulk behavior and single events might not be detectable since they show only a small contribution to the overall melting. This needs attention in discussing melting processes in biological membranes. In the case of biological membranes it might even be difficult to distinguish the melting processes from the baseline of the calorimetric scan. This means that melting is present, but it cannot be detected at all using DSC.

FTIR: The Two Approaches

In this paper we used two different techniques to analyze the FTIR data. These were following the temperature dependence of the absorption maxima of either the symmetric CH_2 or the asymmetric CD_2 stretch vibrations and the area change of one of the peaks of a difference spectra.

Using the first method we found , in accordance to (28), that the absorption maximum of the CH_2 stretch vibrations of the *DSPC* lipids developed towards smaller wavenumbers when the *DMPC-d54* started to display stronger fluctuations (see the right panels of fig. 3). It has been interpreted that in these cases the *DSPC* lipids start to order. Concluding from the data presented in this work and by Leidy et al. (28) this ordering should be stronger than in the presence of a complete *solid ordered* membrane. The wavenumber at which the absorption is at a maximum drops to wavenumbers even smaller than at low temperatures. There is, however, no further supporting evidence that lipids might be able to order stronger than at low temperatures. This behavior is in disaccord of what we have found with the second method and the Monte Carlo simulations. Therefore, it seems reasonable that the Difference Spectra method yields results which describe the melting process correctly and monitoring band shifts only can lead to incorrect results.

Biological Relevance

The model of a biological membrane by Singer and Nicolson considers the biological membrane to be homogeneous (71). In the recent years this view has changed. A heterogeneous membrane model has overtaken this older picture (72, 73). Different kinds of heterogeneities can be present in biological membranes, such as protein aggregates or lipid domains. Even if a membrane should be completely in the *liquid disordered* phase, lipids might not mix ideally and a heterogeneity exists as seen in fig. 1 and as already discussed elsewhere (57). This statement is also true for membranes in the *liquid ordered* phase (74). The role of lipid membrane heterogeneities has been discussed exhaustingly in the literature (25, 34, 38, 39, 40, 41, 43, 44, 45, 46, 47). Thereby, it has not only been pointed out that the coexistence of solid ordered and liquid disordered phases might be important (39, 40, 41, 43, 44), but also the enrichment of single lipid species in certain regions of the lipid membrane (42, 45, 46, 47).

Melting of single components cannot be detected from the bulk melting in the calorimeter. However, most of the publications cited here deduce phase separation of natural membranes from calorimetric results. Therefore, details of these processes might not be revealed. It is also possible that the melting process in a biological membrane displays a broad profile and the heat changes detected by a calorimeter cannot be distinguished from the background signal.

In this context not only the various lipid membrane phases have to be named, but also the possibility of enrichment of certain lipid species in the certain lipid domains. Therefore, however, a detailed understanding of lipid melting transitions and lateral structuring need to be obtained. The existence of heterogeneities depends on the thermodynamical behavior of the different lipid species. As seen in this study they melt over a broad temperature regime with particular temperatures at which they display strong fluctuations in more complex lipid mixtures. These temperatures depend on the lipid species, but also on its interactions with other kinds of lipid present. Melting processes might not be finished even though a heat capacity profile might not indicate any such events. The sensitivity might be not sufficient. FTIR spectroscopy, however, provides means to distinguish the melting of single components.

A possible reason for the big variety in lipid species might be to ensure a needed lipid membrane heterogeneity and therewith to play a role membrane function. This then provides an explanation why a dependence of lipid synthesize in biological membranes on outer physical parameters such as pressure or temperature is required.

Acknowledgments

M. Fidorra was funded by BioNet and the Villum Kann Rasmussen Foundation.

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