Structural characterization of multi-lamellar lipid bilayers and lipid/tri-block copolymer systems by small-angle neutron and x-ray scattering.

by

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## II Structural and thermal characterization of ternary systems of di-myristoyl-phosphatidyl-choline, PEO-PPO-PEO tri-block copolymer (Pluronic® P85) and salt solution 77

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Abstract

In the present ph.d. thesis two aspects of the lipid system are being studied. The first part focus on the most optimal way to obtain structural information from small-angle neutron or x-ray scattering spectra, performed on fully hydrated pure lipid systems. The second part contains experimental studies, performed on DMPC/P85/solvent systems, in which the effect of P85 is being determined.

In Part I the small-angle neutron and x-ray spectra are being analyzed by different models of the scattering function. These models are based on two different theories; the paracrystalline and Caillé theory, and three different models of the scattering length density profile. In order to take the methyl-terminus in the middle of the bilayer into account the Gaussian fluctuating strip model by Lemmich et al. has also been expanded. Part I does not give an unambiguous answer to the most optimal combination of theory and scattering length density profile model to use when obtaining structural information from small-angle neutron and x-ray scattering spectra. The result is mainly caused by large differences in the fitting capability of the scattering function models.

In Part II the DMPC/P85/solvent system is being studied by small-angle x-ray and differential scanning calorimetry. The experimental studies show that P85 has an effect on the aggregation of DMPC, which e.g. can be seen from the appearance of the bicontinuous cubic phase, Pn3m, for temperatures below the main-phase-transition. A similar phase can not be observed in pure DMPC and P85 systems. From the differential scanning calorimetry studies it can furthermore be seen that lipid molecules exist with both low and high cooperativity between the acyl-chains. Finally, small-angle x-ray studies, where P85 is being replaced with PEG, show that the effect of P85 is not only due to the presence of PEO-blocks in the system, but also the interaction between the PPO-block and the lipid molecules.
Resumé

I denne ph.d. afhandling behandles 2 aspekter af lipid systemer. Den ene del fokuserer på, hvordan man mest optimalt opnår strukturel information fra små-vinkel neutron og røntgen sprednings data, udført på fuldt hyderet rene lipid systemer. Den anden del består af en række eksperimentelle studier, udført på DMPC/P85/salt opløsnings systemer, hvor det forsøges klarlagt om Pluronic® P85 har en effekt på aggregeringen af systemet, og i givet fald hvilken.

I Part I analyseres små-vinkel neutron og røntgen sprednings data ved hjælp af forskellige modeller for spredningsfunktionen. Disse modeller er baseret på to typer af teorier, henholdsvis parakrystallinsk og Caillé teori, samt forskellige modeller for dobbeltlagets sprednings længde tætheds profil. Lemmich et al. gaussisk fluktuerende strip model er i denne sammenhæng blevet vidreudviklet, for at tage hensyn til acyl-kædernes metyl-ender i midten af dobbeltlaget. Part I giver ikke et entydigt svar på hvilken kombination af teori og model for sprednings længde tætheds profilen, der er mest optimal til at udlede strukturel information fra små-vinkel neutron og røntgen sprednings data. Resultatet skyldes primært en stor forskel i modellernes evne til at fitte til de forskellige eksperimentelle data.

I Part II studeres DMPC/P85/salt opløsnings systemer ved hjælp af små-vinkel røntgen spredning sammen med differential skanning kalorimetri. De eksperimentelle studier viser, at P85 har en effekt på aggregeringen af DMPC, hvilket bl.a. ses ved den bikontinuere kubiske fase, Pn3m, som optænder ved temperaturer under hovedfaseovergangen og som hverken fremkommer i rene DMPC eller P85 systemer. Fra DSC studier kan det endvidere ses, at der eksisterer lipid molekyler med både høj og lav kooperation mellem acyl-kæderne. Endelig bekræftet SAXS studier, hvor P85 erstattes med PEG, at P85 effekten på DMPC aggregaterne ikke alene skyldes tilstedeværelsen af PEO-blokke i systemet, men også vekselvirkning mellem PPO-blokken og lipid molekylerne.
Summary and Outline

The original subject of present ph.d. thesis is a study of self-assembly structures of lipids interacting with third components, and this very broad wording has given me a high level of influence in defining the subject.

The Pluronic® P85 has in previous studies shown ability of steric stabilizing liposomes, which is of both physiological and medical interest due to liposomes potential as drug carriers. Further structural and thermodynamically studies of the DMPC/-P85/solvent system are therefore of great interest and leads to an interest of continuing the work initiated by Lemming in comparing one-dimensional models appropriate for structural characterization of multi-lamellar lipid bilayers.

My first intention was therefore a comparative study of theories and models appropriate for structural characterization of multi-lamellar bilayers obtained by small-angle neutron and x-ray scattering and use the results from this study in analyzing scattering data obtained on DMPC/P85/solvent systems.

From a first point of view it seemed natural first to determine an optimal theory and model and then apply them to an experimental study. The unpredictable nature of science results however in very interesting and unexpected observations for the DMPC/-P85/solvent system at P85 weight fractions of $\sim 10$ wt%. The unexpected system behavior, identified as a phase transition bringing the system from a lamellar phase to a cubic phase, leads to an increased and changed focus in understanding the behavior of the DMPC/P85/solvent system in the area around the cubic phase.

Adapting results from the comparative study in the analysis of DMPC/P85/solvent systems, e.g. by fitting the models to the scattering spectra, were therefore no longer relevant, since the theories and models compared are only appropriate for lamellar-phases. This is also why the present ph.d. thesis ends up in two individual parts instead of two strongly connected parts.

**Part I**  In our attempt to find the most appropriate theory and model for structural characterizing of multi-lamellar bilayers, when performing fits on small-angle neutron (SANS) and x-ray (SAXS) scattering, we did not find a clear answer.

In SANS, paracrystalline theory with absolute widths of the layer thickness distribution obtains slightly, but not significantly better fits than special Caillé theory. The comparative study also shows that Caillé theory is best in fitting the region between the diffraction peaks, which interpret the harmonic description underlying Caillé theory on a short-length scale is more appropriate than the stochastic description underlying paracrystalline theory.
In SAXS, the results are clear, since Caillé theory performs significantly better fits than both paracrystalline and decoupled paracrystalline theory, whereby the harmonic description again is most appropriate in describing the multi-lamellar bilayer system.

By comparing the SANS and SAXS data we also found out that the diffraction peaks in SANS data are Gaussian shaped, while they follow a power-law behavior in SAXS. The Gaussian shape can be explained by smearing due to the instrumental geometric set-up and wavelength spread, but might also be the reason why paracrystalline theory adequately describes the fully hydrated multi-lamellar lipid bilayer systems in SANS a little better.

Regarding the scattering length density profile model, 3GSM is recommended when performing fits to SANS spectra. Thus, it is not possible to obtain stable fits with 4GSM and GM, which might be due to contrast matching by deuterium. For SAXS spectra no significant differences in fits performed with 3GSM and 4GSM have been observed, 3GSM is however recommended as model due to the lower number of free structural parameters required. When fitting modified Caillé theory to SAXS spectra the GM model is also a suitable alternative, since GM performs good fits and requires the same number of free structural parameters as 3GSM.

Based on the results from the comparative study, special Caillé theory together with the 3GSM model is suggested as an overall model for the $L_{\alpha}$-phase, since this combination obtains fits of high quality when performed to both SANS and SAXS spectra. For all other phases paracrystalline theory and 3GSM is a more appropriate combination.

In general, there is no evidence for a change in above mentioned results when applying a third component to the bilayer system, as long as the behavior of the bilayer is not significantly changed. This means the third component should only interact with the bilayer through entering and without causing any structural changes, because when in-plan ordering starts forming then none of the models are able to adequately describe the bilayer system due to their one-dimensional nature. In relation to P85, the copolymer was originally expected to slightly destabilize the DMPC bilayer at low weight fractions, which will increase the disorder of the system and be reflected in the structural parameters as increasing value of the fluctuation parameters. However, due to change in focus none of the theories have been used in the experimental studies in Part II whereof we are not able to make further conclusion on the influence of incorporating P85 to the multi-lamellar lipid bilayers.

In relation to structural characterization of the lipid bilayer system, the results obtained by SANS and SAXS are somewhat not comparable due to differences in fitting parameters and parameter values. However, by taking e.g. the difference in instrumental set-ups and chemical knowledge about lipid molecules into account the results become similar and acceptable, since the thickness variables for all theories and models are within statistical error. The structure of the bilayer is analyzed in relation to thickness variables such as the thickness of the hydrophobic core, the bilayer thickness and the total size of fluctuations within the bilayer, and the obtained results from the SANS and SAXS scattering data are despite differences in definition in reasonable agreement with parameter values obtained by other groups, e.g. Nagle and Tristram-Nagle (2000) and G. Pabst and Rappolt (2003).
From the analysis it can e.g. be seen that the thickness of the hydrophobic core is larger for the \( L_{\beta'} \) and \( P_{\beta'} \) gel-phases when compared to the fluid \( L_{\alpha} \)-phase, and that 4GSM in general obtains larger values for the hydrophobic core than 3GSM. From the fluctuation parameters it can also be seen that the bilayer fluctuates less in the \( L_{\beta'} \)-phase than in the \( L_{\alpha} \)-phase, which is expected when going from an ordered gel-phase to a less ordered fluid-phase. In comparison, the fluctuations of the bilayer are larger for the \( P_{\beta'} \)-phase than the \( L_{\alpha} \)-phase, which is against our expectations and might be due to the fact that the ripple structure is not taken into account in any of the theories and models, which leads to an overestimation of the fluctuation values of the bilayer.

Finally, it can be concluded that the physical assumption about fluctuations of the different parts of the bilayer being relative to the thickness of the respective parts of the multi-lamellar bilayer is only valid for decoupled theories. This leads to the conclusion that the size of the water layer fluctuation is independent of the size of the fluctuations within the bilayer.

**Part II** The structural and thermal characterization of DMPC/P85/solvent systems shows that ternary systems containing 30 wt.% solvent have a different behavior than systems with a higher solvent content. The differences can be seen at both 20°C and 35°C and various P85 weight fractions. The phase at 20°C seems to be a stabilized \( P_{\beta'} \)-phase, and it might be similar to the phase observed in DMPC/cholesterol systems Mortensen et al. (1988). At 35°C, the system exists in a lamellar phase similar to the \( L_{\alpha} \)-phase of the pure aqueous DMPC/solvent system for P85 weight fractions up to 40 wt%.

For samples with \( w_{P85} = 10 \) wt.% and dissolved to a solvent content of 70 wt% the DMPC/P85/solvent system is in agreement with the Pn\( \bar{3} \)m cubic symmetry. In this Pn\( \bar{3} \)m phase, the lipids might arrange themselves in rods with a radius equal to half of the bilayer thickness as proposed by Funari et al. (1997). Above the main-phase-transition temperature, the system is in a lamellar phase, and the repeat distance for this lamellar phase is significantly higher than the corresponding lamellar \( L_{\alpha} \)-phase of the pure aqueous DMPC/solvent system. The repeat distance increases with increasing temperature, which might be caused by the PEO-segments or the entropic effect.

From the differential scanning calorimetric study it is shown that a pre- and main-transition are preserved in the system, for samples with \( w_{P85} = 10 \) wt.% and dissolved to a solvent content of 70 wt%, which indicates the bilayer structure is present in the system. The main peak seems to split up into two peaks, one peak with high cooperativity, determined as the main-transition, and one rather broad peak with low cooperativity appearing at slightly higher temperatures. The presence of a sharp enthalpy peak with high cooperativity reflects that the mutual orders between the acyl-chains of the lipid molecules are preserved in the low-temperature non-lamellar cubic Pn\( \bar{3} \)m phase. The broad enthalpy peak appearing just above the main-phase-transition is on the other hand designated to chain melting of lipid molecules, which reflects DMPC forming complexes with P85 or at least having P85 in the direct neighborhood, indicating a kind of phase separation on the molecular scale. This phase transition could be similar to the one suggested for DMPC bilayer systems incorporated with cholesterol (Mortensen et al., 1988).
Based on the results obtained by temperature scan, one might speculate in some in-plane ordering within the lamellaes, similar to the properties found in gel-phases of DMPC vesicles incorporated with cholesterol, or the more plausible explanation that some kind of coexistence between the lamellar-phase and another phase exists.

Finally, the comparison study with PEG homopolymer shows that P85 is incorporated into the bilayer, which makes P85 a candidate for steric stabilizing liposomes. When using liposomes as drug carriers, attention should from a medical point of view be paid to the phase transitions occurring in the DMPC/P85/solvent system even at low P85 weight fractions, since they causes a risk of breaking apart the liposomes or losing the liposomes ability to encapsulate a drug.
Abbreviations

CMC  Critical Micelle Concentration
CMT  Critical Micelle Temperature
CT   Caillé Theory
DMPC Di-Myristoyl-Phosphatidylcholine
DMPC-d54 DMPC with fully deuterated acyl-chains
DPPC Di-Palmitoyl-Phosphatidylcholine
DPPC-d62 DPPC with fully deuterated acyl-chains
DPT  Decoupled Paracrystalline Theory
DSC  Differential Scanning Calorimetry
EO   Ethylene Oxide
GM   Gaussian Model, consisting of two head groups and one methyl-terminus layer
3GSM 3 Gaussian Strip Model, consisting of two head groups, two acyl-chain layers and one water layer
4GSM 4 Gaussian Strip Model, consisting of two head groups, two acyl-chain layers, two methyl-terminus layers and one water layer
MCT  Modified Caillé Theory
NMR Neuclear Magnetic Resonance
P85  The Pluronic® EO25PO40EO25 tri-block copolymer
PC   Phosphatidylcholine
PEG  Poly Ethylene Glycol
PEO  Poly Ethylene Oxide
PO   Propylene Oxide
PPO  Poly Propylene Oxide
PT   Paracrystalline Theory
SANS Small-Angle Neutron Scattering
SAXS Small-Angle X-ray Scattering
SCT  Special Caillé Theory
subscript ‘r’ The widths of the layer thickness distribution are relative
subscript ‘a’ The widths of the layer thickness distribution are absolute
subscript ‘d’ The diffuse term are included
Chapter 1

General introduction

The biological membrane of all organisms is a very important component for the structure and function of the living cells. Every living cell has a biomembrane (plasma membrane) that serves as a highly selective semi permeable barrier enclosing the cell. The plasma membrane controls to a high extend the flow of chemical spices in and out of the cells. In eukaryotic cells the biomembrane also acts as border for different organelles within the cells that serve different functions, like mitochondria and ectoplasmatic reticulum. The number of different spices in the biomembrane are enormous, and the composition varies a lot. Basically, biomembranes consist of lipids and protein molecules. The lipids form a bilayer sheet that serves as a matrix for the proteins (Singer and Nicolson, 1972). Under most physiological conditions the lipid molecules are able to diffuse around each other like a two-dimensional liquid (Singer and Nicolson, 1972). The physical condition of the lipid bilayer plays an important role in the function of the proteins in the biomembrane. Sessa and Weissman have suggested to use phospholipid liposomes as a model membrane (Sessa and Weissman, 1968).

The present ph.d. thesis focus on two different and independent aspects of phospholipids. One aspect is how to extract structural information from small-angle neutron and x-ray scattering spectra. The second aspect is a experimental study of how Pluronic® P85 influences the aggregation of DMPC in a solvent system.

1.1 Phospholipids

The major structural component of biological membranes are the lipids. The main groups of lipids include phospholipids, glycosphingolipids and cholesterol. Phospholipids can be categorized in two major groups, phosphodiglycerides and sphingolipids (Figure 1.1). Phosphatidylcholine also denoted PC (Figure 1.2) is the most common group of phospholipids and belongs to the major group of phosphodiglycerides. The importance of phospholipids to living cells, can be pointed out by the nearly complete lack of genetic defects in the phospholipids metabolism in humans. Such defects are presumably lethal at an early state and have never been discovered (Zubay, 1993a).
CHAPTER 1. GENERAL INTRODUCTION

Chemical structure

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<td>1,2-diacyl-sn-glycerol-3-phosphatidyl choline</td>
</tr>
<tr>
<td><img src="image2.png" alt="Chemical structure" /></td>
<td>N-acyl-trans-4-sphingenie-1-phosphoryl choline</td>
</tr>
</tbody>
</table>

Figure 1.1: The figure shows the two main groups of phosphocholine lipids. Phosphocholine lipids are the most abundant lipids in nature.

Figure 1.2: The figure shows the structure of phosphatidylcholine. The positive choline head group and the negative phosphate group make phosphatidylcholine zwitterionic. Together with the hydrophobic fatty acids attached to position 1 and 2, the charged head group and phosphate group give the phosphatidylcholine molecule it’s amphiphilic properties. (Figure adapted from Alberts et al., 1997)

1.1.1 Phosphodiglycerides

Common to all phosphodiglycerides are the glycerol-3-phosphate backbone. The two hydroxy groups on carbon 1 and 2 take part in ester bonds with fatty acids. The naming of the phosphoglycerides depends on which head group there is bond to the phosphate group. In Figure 1.3 some of the common naturally occurring head groups of the phosphodiglycerides are shown. The fatty acids found from natural sources have almost without exception always had an even number of carbon atoms in the chain. This is a consequence of how the lipids are biosynthesized. The lipids are built up by adding acetyl-CoA one by one in each cycle. After at least 16 carbons have been reached, desaturation can occur as shown in Figure 1.4. Most of the naturally occurring phospholipids are 'mixed', which means the fatty acid chain attached to position 1 is different from the one attached to position 2 (Zubay, 1993a; New, 1990a).

1.1.2 Morphology of lipid aggregates in water

The phospholipids are amphiphilic molecules with a hydrophilic head group region and a hydrophobic fatty acid chain region. The fatty acid chains, often called the hydrocarbon tails or just lipid chains, vary in number of carbons in the chain with an even number of carbon atoms and degree of saturation.

The lipid chains make the phospholipids insoluble in water in the accepted way.
### 1.1. PHOSPHOLIPIDS

<table>
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<th>Headgroup</th>
<th>Common name</th>
<th>Abbreviation</th>
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<td>Phosphatidyl choline</td>
<td>PC</td>
<td>choline</td>
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<tr>
<td>Ethanolamine</td>
<td>PE</td>
<td>ethanolamine</td>
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<tr>
<td>Serine</td>
<td>PS</td>
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<tr>
<td>Glycercol</td>
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<td>Acid</td>
<td>PA</td>
<td>acid</td>
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<tr>
<td>Inositol</td>
<td>PI</td>
<td>inositol</td>
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Figure 1.3: Phosphodiglycerides are categorized depending on the radical (head group) bond to the phosphate group. The figure shows some of the common naturally occurring phosphatidyl phosphodiglycerid head groups. Except for PC and PE, all phosphatidyl lipids are negatively charged, since the negative charge on the phosphate is not balanced out by the head group.

Figure 1.4: The figure shows the biosynthetic pathways for some natural monounsaturated fatty acid chains (adapted from New, 1990).

In the aqueous medium membrane lipids will, even at very low concentrations, self-assemble into different aggregates with the hydrophilic head groups exposed to the aqueous medium, and the hydrophobic hydrocarbon tail shielded against the aqueous medium. The main driving force for this aggregation is the entropic effect. Exposure of a hydrophobic hydrocarbon tail to water will cause an unfavourable decrease in entropy, which will order the water molecule in a lattice-like structure around the hydrocarbon tail due to the lack of hydrogen bonding to the hydrocarbon tail (Zubay, 1993). This effect is normally referred to as the hydrophobic effect (Tanford, 1980).

The aggregates formed by the phospholipids mixed with water depend on the shape of the lipids, the water content and the temperature, and range from small micelles, different lamellar phases, vesicles, hexagonal rods, inverted hexagonal rods and several cubic phases. The phospholipids with the position 2 hydrolyzed, the so called lysopho-
phospholipids, form especially small micelles (Figure 1.5d) because the cross-section of the head group is larger than the cross-section of the hydrophobic tail. The lipids arrange themselves so the hydrophilic head groups are in contact with the water and shield the hydrophobic tails, which are pointing inwards from the water. At the water-air inter-

![Image](a) Ribbon phase, $P_\sigma$

![Image](b) Hexagonal phase, $H_I$

![Image](c) Hexagonal phase, $H_{II}$

![Image](d) Micelle

![Image](e) Cubic bicontinuous primitive (P) phase, $Q^{229}$ or Im$\bar{3}m$

![Image](f) Cubic phase, $Q_\alpha$

![Image](g) Lamellar phase, $L_\alpha$

![Image](h) Vesicles

Figure 1.5: Phospholipids suspended in water form aggregates with different morphologies, depending on the concentration and composition of the phospholipid together with the temperature. At low lipid concentration DPPC forms bilayer vesicles, but with increasing concentration there will however not be enough water to form vesicles and the bilayer will therefore break-up and form bilayer sheets. At even higher concentrations, phospholipids can form different cubic and hexagonal phases.

...face the lipids are able to form a lipid monolayer with the hydrophobic hydrocarbon tails pointing out in the air and the hydrophilic head group remaining in contact with the water (Zubay, 1993a). The hexagonal phases exist in two versions; hexagonal rods ($H_I$, Figure 1.5b) and inverted hexagonal rods ($H_{II}$, Figure 1.5c). Both phases consist of cylindrical long rods of lipids arranged in a hexagonal array. $H_I$ is of the type where...
the hydrophobic tails point towards the center of the rod, and \( H_{II} \) is of the type, in which the head group forms channels filled with water (Small, 1986a,b). Several cubic phases have been found for lipid water systems. In the di-palmitoyl-phosphatidylcholine (DPPC) water system, at low water content and high temperature, a cubic phase denoted \( Q_\alpha \) is found (Sachmann, 1983). In this phase the water is dispersed in a matrix of the hydrocarbon tails with the head groups align in the interface of the water and lipids (figure 1.5f). Other cubic phases have been found for lipids, one of these is the bicontinuous primitive (P) phase represented by the Im\( \bar{3} \)m or \( Q_{229} \) space groups (Figure 1.5e). The phase consists of two water channel systems, which are separated by the lipid bilayer (the phase is also denoted "Plumber’s nightmare") (Rummel et al., 1998). For the DPPC water system at very low water content, a ribbon phase, \( P_\sigma \), exists (Figure 1.5f). With increasing lipid fraction different lamellar bilayers are forming. Common for these lamellar bilayer phases are the consistency of two parallel monolayer sheets. Within the individual monolayer lipids align head group against head group and tail against tail, and the two sheets arrange so the hydrophilic head group is exposed to the water and forms boundaries between the water and the hydrophobic hydrocarbon tails (Figure 1.5g). Above a certain water content, called the swelling limited, the lamellar phases of phosphatidylcholine co-exist with a bulk water phase or excess water phase. In these phases the bilayers enclose themselves to form vesicles. The bilayer stops swelling at a certain level of water indicating that the phosphatidylcholine bilayer is bound together with a well defined repeat distance.

1.1.3 Phase transition

Binary phospholipid/water systems are able to undergo phase transitions depending on temperature and water content. The phase transition occurs not only between main structural different phases, but also between different lamellar phases (Figure 1.6a). Three major first-order transitions are found for the phosphatidylcholine bilayer (Small, 1986c) and denoted the sub-, pre- and main-phase-transitions, which correspond to four different lamellar phases. At low temperature the phosphatidylcholine bilayer exists in a crystalline lamellar phase, \( L_c \) or \( L_c' \), in which the lipid molecules are arranged into a crystalline two-dimensional orthorhombic hybrid lattice plane with a specific chain-chain packing, and with the fatty acid chains ordered in a trans conformation. Out of plane the bilayers are ordered in a one-dimensional array (Small, 1986c), and in this phase the chains are nearly normal to the bilayer plane. After increasing the temperature the bilayer will undergo a sub-phase transition which brings it to the \( L_{\beta'} \) phase (Figure 1.6b), a so called "Gel-phase", where the chains are packed in a disordered two-dimensional rectangular lattice, i.e. as a distorted hexagonal lattice. The chains are tilted an angle, \( \theta_t \), which depends on the temperature, water content and type of fatty acid chains. The tilt is caused by the mismatch in the cross-section of the hydrophobic tails and the hydrophilic head groups. Between the pre-transition and main-phase-transition, the bilayer is in the ripple "Gel" phase, \( P_{\beta'} \) (figure 1.6c). The pre-transition results in a re-arrangement of the lipid molecules from distorted hexagonal packing to a true hexagonal packing. Furthermore the phase transition introduces rippling of the bilayer structure out of plane. Depending on the water
CHAPTER 1. GENERAL INTRODUCTION

Figure 1.6: (a) shows the temperature versus water content phase diagram of DMPC. At low temperature and water content, the lipids order in a crystalline lamellar phase (L). With increasing temperature the bilayer undergoes different phase transitions which brings it through "gel" phases (L$_{\beta'}$ and P$_{\beta'}$) and ends up as a two dimensional liquid (L$_{\alpha}$). At temperatures between 25 and 35°C and low water content, DMPC forms a hydrated crystalline phase with a orthorhombic cell (C). (b,c,d) show the structures of the L$_{\beta'}$, P$_{\beta'}$ and L$_{\alpha}$-phases. In the L$_{\beta'}$-phase the hydrocarbon chain packing is a "distorted" hexagonal lattice, whereas for the P$_{\beta'}$-phase the chain pack in a hexagonal array. For both phases the acyl chains are in an all trans conformation. In the L$_{\alpha}$-phase the chain is melted and the molecules are not fixed in an array.

content the ripple period varies by around two or three times the repeat distance (Small, 1986c). At the main-phase-transition the chains go through the transition from ordered hexagonal packing to a melted disordered state, L$_{\alpha}$ (Figure 1.6d), with a high degree of freedom. In this phase the bilayer is a planar (the ripples disappear) two-dimensional fluid and with the lipid molecules free to move around. The melting of the acyl-chains and the loss of lateral crystalline structure, as the system passes from the P$_{\beta'}$-phase to the L$_{\alpha}$-phase, gives rise to a decrease in the bilayer thickness and a concomitant increase in the bilayer area (Kinnunen and Laggner (Eds.), 1991). The main transition is a weak first-order transition (Mouritsen, 1991), i.e. close to a critical point, which implies the transition is accompanied by strong in-plane density fluctuations. These fluctuations give rise to an effective decrease in the bilayer bending rigidity (Hønger et al., 1994; Lemmich et al., 1994; Hansen et al., 1997), which cause the bilayers to exhibit strong out-of-plane undulations. The three phases L$_{\beta'}$, P$_{\beta'}$ and L$_{\alpha}$ are lyotrophic liquid crystals and can be divided into a smectic B state (L$_{\beta'}$ and
1.1. PHOSPHOLIPIDS

$P_{\beta'}$ and a smectic A state ($L_\alpha$) (Small, 1986b).

Common to all phospholipid bilayers are the exhibition of the $L_c$, $L_\beta$ and $L_\alpha$-phases, as described for phosphatidylcholine. If the chains are tilted, the subscript is marked with a prime like $L_{\beta'}$. Phosphatidylethanolamine also exhibits the additional ripple phase $P_{\beta'}$ like phosphatidylcholine. The phase transition temperature of the phosphodiester lipid depends on the length of the fatty acid chains, the degree of saturation and the type of head group. (Figure 1.7).

![Figure 1.7: The figure shows the main-phase-transition temperature versus chain lengths for diacyl phosphoglycerides with different head groups. The pre-transition temperature is also shown for phosphatidylcholine. The two fatty acid chains are of the same chain length, and in case of unsaturation, the double bonds are at the same position for both fatty acid chains. All the monounsaturated acids have the double bond at position 9, in either cis or trans conformation indicated by a superscript 'c' or 't'. The Figure is adapted from New (New, 1990b)]](image)

1.1.4 Experimental techniques

Common used methods to study lipids and biomembranes are Small-Angle Neutron Scattering (SANS) and Small-Angle X-ray Scattering (SAXS). These techniques are valuable in both structural characterization as well as studies of phase transitions and phase diagrams. To support SANS and SAXS experiments, Differential Scanning Calorimetry (DSC) is very useful, since DSC is one of the strongest techniques to obtain thermodynamic parameters, giving strong indications of phase transitions.

1.1.5 Restrictions

In Part I the ph.d. thesis is restricted to spectra obtained on two kinds of phosphatidylcholine lipids, DMPC and DPPC. The analysis is furthermore restricted to the two basic scattering theories: paracrystalline theory and Caillé theory.
In Part II the experiments are restricted to only be performed on DMPC phosphatidylcholine lipids and Pluronic® P85 tri-block copolymers. Models fitted to scattering data of DMPC/P85 are neither included in the thesis due to the changed focus and time limits.

Other third components than P85 and PEG 1000/4000 have not been studied in the present ph.d. thesis due to lack of time. In relation to this is should be noticed that the presence of a third component incorporated in the lipid bilayer system normally tends to destabilize the bilayer. This will normally cause an increasing disorder, reflected by larger values for the fluctuation parameters. Some third components are however able to stabilize the bilayer in certain phases, e.g. will cholesterol enter the bilayer in the $L\alpha$-phase and tend to stabilize the gel phases. In other cases the formation of multi-lamellar bilayer systems can be disrupted as illustrated in our studies by the presence of the cubic phase.

1.2 Overview

The present ph.d. thesis consists of two individual parts. The first part focus on numeric and comparative analysis of pure lipid systems, where we try to determine the theory and model which is most appropriate for structural characterization of multi-lamellar lipid bilayer system. The second part is an experimental study of the DMPC/P85/-solvent system.

**Part I:** The basis of Part I comes from a paper of Lemmich et al. (Lemmich et al., 1997). This paper is however only performed on SANS spectra, and does not include a more detailed analysis of the models. This is why we have made an in-depth analysis of the paracrystalline and Caillé theories and the different models for scattering length density profiles - on both SANS and SAXS spectra.

Chapter 2 is devoted to theory, and includes derivation of different scattering functions based on either paracrystalline or Caillé theory and different models for the scattering length density profiles.

In Chapter 3 the results obtained by fitting the different theories and models to SANS and SAXS spectra are analyzed and discussed. The fits to SANS and SAXS are first treated separately, and for each type of spectra a comparison of the different theories and models are performed. Secondly, the results from SANS and SAXS are compared and discussed followed by a general conclusion for all the different theories and models.

**Part II:** This Part is dedicated to structural and thermal characterization of the ternary DMPC/P85/solvent system, mainly obtained by SAXS.

Chapter 4 gives a general introduction to drug delivery systems and the Pluronic® P85 tri-block copolymer.

Chapter 5 analyze and discuss experimental measurements based on DMPC mixed with P85 dissolved in different solvents. For comparison, experiments with P85 being replaced by homopolymer (PEG) have been carried out. The DMPC/P85/solvent system is further more studied regarding DSC and temperature scan and from the different results a general conclusion on the DMPC/P85/solvent system is made.
Part I

Modelling of lipid bilayers
Chapter 2

Theory

Structural characterization of simple lipid bilayers are important for understanding the function of biological membranes (Bloom et al., 1991; Kinnunen and Mouritsen (Eds.), 1994) and has attracted significant attention for several decades. One of the most extensively studied class of lipids are the non-charged di-acyl-phosphatidylcholines.

Luzzati and coworkers were early pioneers in the field of structural characterization of different phases of di-acyl-phosphatidylcholines (Tardieu et al., 1973; Luzzati, 1967). To extract structural information Luzzati used a very simple one-dimensional model assuming that the repeat distance, $d$, can be separated into, a bilayer thickness, $d_B = \varphi_L d$, and a water thickness, $d_W = d - d_B$, where $\varphi_L$ is the volume fraction of the lipid which can be derived from the weight fraction of the lipid, $c_L$, and the specific volumes of water, $\nu_W$, and lipid, $\nu_L$. Together with the molecular weight of the lipid, $M_L$, and the water, $M_W$, Luzzati was able to determine the molecular cross-section, $A$, and the number of water molecules per lipid molecule, $n_W$,

$$A = \frac{2M_L\nu_L}{N_A d_B} = \frac{2M_L}{N_A d} \left[ \frac{1 - c_L}{c_L} \nu_W \right], \quad n_W = \frac{1 - c_L}{c_L} \frac{M_L}{M_W},$$

(2.1)

The Luzzati method assumes that the lipid bilayers form periodic layers together with water, and with no excess water present. Janiak et al. (1976) have also tried to extend the method to include fully hydrated systems, the one-dimensional model do however not take major defects introduced to the system close to the swelling limit and formation of bulk water reservoirs even before the swelling limit has been reached into account, which is why the Luzzati method is unsuitable for fully hydrated systems.

Since Luzzati’s original work, a large number of neutron and x-ray scattering studies have been carried out in order to obtain structural insight. The studies have e.g. been performed on multi-lamellar lipid bilayers of di-acyl-phosphatidylcholines in the $L_{\beta'}$, $P_{\beta'}$, and $L_{\alpha}$-phases and at various degrees of hydration Tardieu et al. (1973); Luzzati (1967); Janiak et al. (1976); Torbet and Wilkins (1976); Inoko and Mitsui (1978); Büldt et al. (1979); Franks and Lieb (1979); Janiak et al. (1979); Zaccai et al. (1979); Lis et al. (1982); King and White (1986); McIntosh and Simon (1986); Smith et al. (1987); Nagle and Wiener (1989); Wiener and White (1991a,b); Kirchner and Cevc (1993); Tristram-Nagle et al. (1993); Kirchner and Cevc (1994); Sun et al. (1994); Gordeliy et al. (1996); Lemmich et al. (1996); Nagle et al. (1996); Sun et al. (1996); Chen et al. (1997).
CHAPTER 2. THEORY

The liquid crystalline nature of fully hydrated multi-lamellar lipid bilayer vesicles can be taken into account by applying paracrystalline theory (Lemmich et al., 1996) and Caillé theory (Zhang et al., 1995; Pabst et al., 2000) to the analysis. Both scattering theories are considered appropriate for analysis of neutron and x-ray data performed on multi-lamellar lipid bilayer systems, but it should be noticed that small-angle neutron and x-ray scattering have different advantages. E.g. an efficient way of exploiting neutron scattering data is using the large difference in the scattering length of hydrogen and deuterium lipids and preparing samples in D$_2$O. In that way a minimum of incoherent (flat) background scattering, $I_{\text{inc}}$, a maximum contrast in the scattering length density normal to the bilayer plane and form factors reaching for high $q$-values can be obtained. X-ray scattering data can not be exploited in the same way, but has the advantage of a higher resolution and a more narrow wavelength spread $\Delta \lambda/\lambda$ than SANS. Some approaches are therefore more suitable for SANS than SAXS and vice versa. Due to the differences in contrast the structural parameters will be different in the analysis of SANS and SAXS scattering data.

Main focus of present ph.d thesis is a comparative analysis of how to obtain the best structural characterization of multi-lamellar lipid bilayers from SANS and SAXS experiments. Special attention will be paid to SANS and SAXS scattering data obtained from fully hydrated multi-lamellar bilayers of di-acyl phosphatidylcholines, DMPC and DPPC in the $L_{\beta'}$ and $L_{\alpha}$-phase and with saturated fatty-acid chain lengths corresponding to 14 and 16 atoms respectively.

Both paracrystalline and Caillé theory and different methods applied to the analysis of scattering data will be critically reviewed.

2.1 Basic Scattering Theory and Fourier Analysis

Even though neutrons are scattered from nuclei and x-ray from electrons, the scattering of both neutrons and x-rays can be described by the scattering length, $b$, of a given atom and the corresponding scattering length density, $\rho$, which is a continuous function. This is valid for low spacial resolutions with scattering vectors $q < 1$ Å$^{-1}$, which are of relevance for SANS and SAXS. It should be noted that for x-ray scattering the scattering length density is proportional to the electron density.

The scattering function, $I(\vec{q})$, for any system which exhibits lamellar structure, can be described as

$$I(\vec{q}) = \langle |f(\vec{q})|^2 s(\vec{q}) \rangle,$$  \hspace{1cm} (2.2)

The $\langle \ldots \rangle$ indicates averaging over all fluctuations in the system, where $f(\vec{q})$ is the form factor of the system and $s(\vec{q})$ is the structure factor. A determination of the form factor makes it possible to extract structural information for the bilayer from either the neutron or x-ray scattering data and is to a good approximation given by the Fourier transformation of the scattering length density profile along the $z$-axis,

$$f(\vec{q}) = f(q_r, q_z) \simeq f_z(q_z) = \int_{\text{repeat unit}} \rho_z(z) e^{-iq_z z} dz \quad \text{when} \quad q_r \ll q_z.$$  \hspace{1cm} (2.3)
As long as the bilayer is nearly flat, \( f(\vec{q}) \) is very small except when \( q_r \ll q_z \) and the approximation is good. According to Eq. (2.3) the scattering length density profile along the \( z \)-axis, \( \rho_z(z) \) can be calculated from the inverse Fourier transformation of the form factor and is given by

\[
\rho_z(z) = \int \Omega f_z(q_z) e^{i q_z z} d q_z \approx \int f(\vec{q}_r, q_z) e^{i q_z z} d q_z \quad \text{when} \quad q_r \ll q_z. \tag{2.4}
\]

In most cases the scattering length density is determined relative to the scattering length density of the solvent. This is true, since adding any constant to the scattering length density will not cause a change in the overall result.

The structure factor, \( s(\vec{q}) \), is defined by the correlation function, which describes the quasi-crystalline nature of a stack of lipid bilayers. By assuming the stack consists of \( N \) lipid bilayers, \( s(\vec{q}) \) is given by,

\[
s(\vec{q}) = \int d^2 \vec{r} d^2 \vec{r}' \sum_{j=1}^{N} \sum_{k=1}^{N} e^{-i \vec{q}_r(\vec{r}-\vec{r}')} e^{-i q_z (z_j-z_k)}, \tag{2.5}
\]

where \( z_k \) denotes the position of the \( k \)'th bilayer in the stack. \( \vec{r} \) and \( \vec{q}_r \) is the direction perpendicular to the bilayer normal in real-space and \( q \)-space. Since there is no evidence of any in-plan asymmetry in the bilayer, the bilayer stack is assumed to be cylindrical with radius \( R \) and area \( A_s \), Lemmich et al. (Lemmich et al., 1997) could, by using translational invariance, write Eq. (2.5) as

\[
s(\vec{q}) = 2 \pi A_s \int_0^R d r r J_0(q_r r) \left[ N + 2 \sum_{k=1}^{N} (N-k) e^{-i q_z z_k} \right] \\
= 2 \pi A_s \frac{R}{q_r} J_1(q_r R) \left[ N + 2 \sum_{k=1}^{N} (N-k) e^{-i q_z z_k} \right], \tag{2.6}
\]

where \( J_0(x) \) and \( J_1(x) \) are the zeroth- and first-order Bessel functions.

Scattering vectors, \( \vec{q} \), which are nearly normal to the bilayer plane, give strong contributions to \( s(\vec{q}) \), when assuming the stack is nearly flat. Therefore \( \vec{q}_r \) will only contribute if \( q_r \ll q_z \). To a good approximation the orientational average over the \( \vec{r} \)-direction does not depend on the \( z \)-direction, and can therefore be treated independently. Using the powder-averaging theorem (Warren, 1990)

\[
\int \int d^2 \vec{q}_r \int dq_z \frac{s(\vec{q})}{q} = 4 \pi \int dq q s(q), \tag{2.7}
\]

within the approximation \( q_r \ll q_z \) and \( q_z \rightarrow q \) for \( qR \gg 1 \), Lemmich et al. (Lemmich et al., 1997) obtained a simple description of the structure factor, \( s(q) \), given by

\[
s(q) = \frac{2 \pi A_s}{q^2} \left[ N + 2 \sum_{k=1}^{N} (N-k) e^{-i q_z z_k} \right] \equiv \frac{2 \pi A_s}{q^2} s_1(q). \tag{2.8}
\]
From Eq. (2.3) it can be seen that by doing the transformation $q_z \to q$, Eq. (2.2) can be rewritten as

$$I(q) = \frac{2\pi A_s}{q^2} I_1(q)$$

(2.9)

with

$$I_1(q) = \langle|f(q)|^2 s_1(q)\rangle.$$  

(2.10)

According to the Debye approximation (Guinier, 1963a), the form and structure factors can be decoupled, if there are no dependencies between the fluctuation within the individual bilayers and the fluctuation in the bilayer position. Lemmich et al. (Lemmich et al., 1997) then arrived at,

$$I_1(q) = |\langle f(q) \rangle|^2 \langle s_1(q) \rangle + N (\langle |f(q)|^2 \rangle - |\langle f(q) \rangle|^2).$$

(2.11)

The last term in Eq. (2.11), which gives rise to a diffuse scattering of low intensity, is often neglected when the $q$-range in the vicinity of the diffraction peaks are considered.

$$I_1(q) = |\langle f(q) \rangle|^2 \langle s_1(q) \rangle \equiv |F(q)|^2 S_1(q).$$

(2.12)

When the bilayers are perfectly arranged in a one-dimensional lattice, it can be seen from Eq. (2.8) that $S_1(q)$ in Eq. (2.12) will obtain the ideal form

$$S_{1, id}(q) = N + 2 \sum_{k=1}^{N} (N - k) \cos(kqd) = \frac{\sin^2(\frac{1}{2}Nqd)}{\sin^2(\frac{1}{2}qd)},$$

(2.13)

which is the classical result. When $N$ is large, $S_{1, id}(q)$ is reduced to a set of $\delta$-functions (Bragg peaks) of equal magnitude, centered around $q_h = 2\pi h/d$, where $h$ denotes the diffraction order. From Eqs. (2.9) and (2.12) it can be seen that $|F(q_h)|^2$ can be determined by integrating the $h$'th peak, but in practice the integration has to be performed in the vicinity of the peak,

$$\int_{q_h-\epsilon}^{q_h+\epsilon} I(q) dq \propto \frac{|F(q_h)|^2}{q_h^2}.$$  

(2.14)

From Eq. (2.3) it can furthermore be seen that $\rho$ can be calculated from the inverse Fourier transformation of $F(q_h)$. For discrete form factors, $\rho$ will be reduced to a summation over all $h$,

$$\rho(z) = \sum_{h} F(q_h) \cos \left( \frac{2\pi h}{d} z \right).$$

(2.15)

In order to use $|F(q_h)|$ in a calculation, the phase factors of $F(q_h)$ have to be known. For symmetric systems like bilayer stacks the phase factors are restricted to only be $\pm 1$, whereas $F(q_h)$ is real. Since consensus seems reached for the phase factors of phospholipid bilayers (McIntosh and Simon, 1986), no further discussion will be made on this subject. Eqs. (2.14) and (2.15) form together with the above mentioned phase information the basis of the Fourier analysis, often referred to as the Fourier method.
2.2 Structure factors

The Fourier method suffers from the very idealized assumption that the bilayers are perfectly stacked without any kind of distortion. By assuming the \( k \)’th bilayer is fluctuating by \( u_k \) around its equilibrium position relative to the origin \( u_0 \), we get

\[
S_1(q) = N + 2 \sum_{k=1}^{N} (N - k) \langle e^{-iqz_k} \rangle
= N + 2 \sum_{k=1}^{N} (N - k) \cos(kqd) \langle e^{-iq(u_k - u_0)} \rangle.
\] (2.16)

Assuming the fluctuations are Gaussian, \( S_1(q) \), can also be written as

\[
S_1(q) = 2 \sum_{k=1}^{N} (N - k) \cos(kqd) e^{-\frac{1}{2}q^2\sigma_D^2} \langle (u_k - u_0)^2 \rangle_{d1}. \] (2.17)

According to Hosemann and Bagchi the fluctuations can be classified as disorder of the first kind and disorder of the second kind (Hosemann and Bagchi, 1962a). In disorder of first kind, e.g. thermal disorder, the bilayer oscillates around well defined positions, which means the system has true crystalline long-range order

\[
\langle (u_k - u_0)^2 \rangle_{d1} = \langle (u_1 - u_0)^2 \rangle_{d1} \equiv \sigma_D^2 = \text{constant},
\] (2.18)

\[
S_{1,d1}(q) = N + 2e^{-\frac{1}{2}q^2\sigma_D^2} \sum_{k=1}^{N} (N - k) \cos(kqd)
= N(1 - e^{-\frac{1}{2}q^2\sigma_D^2}) + e^{-\frac{1}{2}q^2\sigma_D^2} S_{1,d1}(q),
\] (2.19)

where the subscript ‘d1’ denotes disorder of first kind. From the expression, it can be seen that the Debye-Waller factor, \( e^{-\frac{1}{2}q^2\sigma_D^2} \) (Guinier, 1963b), only affects the amplitude of the peaks, whereas it has no effect on the peaks width when the diffraction order, \( h \), increases. The lost intensity appears as diffuse background scattering, which increases asymptotically to \( N \) with increasing \( q \)-values.

Disorder of second kind is a lattice disorder, where the position of each bilayer oscillates relative to the neighboring bilayers and not to an ideal equilibrium position. Because the position fluctuations sums up, the true crystalline long-range order breaks down,

\[
\langle (u_k - u_0)^2 \rangle_{d2} = k\langle (u_1 - u_0)^2 \rangle_{d2} \equiv k\sigma_D^2,
\] (2.20)

\[
S_{1,d2}(q) = N + 2 \sum_{k=1}^{N} (N - k) \cos(kqd)e^{-\frac{1}{2}q^2\sigma_D^2}
= N \frac{1 - e^{-q^2\sigma_D^2}}{1 + e^{-q^2\sigma_D^2} - 2 \cos(qd)e^{-\frac{1}{2}q^2\sigma_D^2}},
\] (2.21)

where the subscript ‘d2’ denotes disorder of the second kind. Again, the amplitude of the peaks decrease with increasing diffraction order, \( h \). But in this case the intensity
are partly recovered by increasing peak widths. The lost scattering intensity is again shown as diffuse background scattering (cf Figure 2.1), and increases asymptotically to $N$ with increasing $q$-values. A structure factor like, $S_{1d2}(q)$, where the disorder is purely of the second kind, is also denoted the paracrystalline structure factor (Guinier, 1963c).

Figure 2.1: The figure shows a comparison of structure factors. The dotted line shows the paracrystalline structure factor, $S_{1d2}(q)$, Eq. (2.21), the fully line the Caillé structure factor, $S_{1,CT}(q)$, Eq. (2.31), and the big dots the discrete Caillé structure factor, $S(q)$, from Zhang et al. Eq. (78) (Zhang et al., 1994)). The parameters are $\eta_1 = 0.03$, $N = 20$, $d = 60\,\text{Å}$ and $\sigma_D = 6\,\text{Å}$, where $\eta_1$ is defined in Eq. (2.26).

Based on the two kinds of disorder it is clear that only when the width of the diffraction peaks do not increase with increasing diffraction order a system can be considered as an ordered crystal, showing disorder of the first kind. The use of Eq. (2.14) in determining form factors for bilayer systems has both been discussed (Wiener and White, 1991a,b; Torbet and Wilkins, 1976; Franks and Lieb, 1979) and reviewed (Blaurock, 1982)).

In terms of disorder of the first and second kind, fully hydrated multi-lamellar bilayer systems are predominated by disorder of second kind, which is shown by the quasi-long range order observed perpendicularly to the bilayer plan. This is also reflected in the shape of the diffraction peaks which are broader for fully hydrated multi-lamellar bilayer systems and increases in width as the diffraction order, $h$, increases. This cause several problems when using the Fourier method. Firstly, the assumption in Eq. (2.14) can only be considered accurate if $F(q_h)$ is nearly constant through the integration interval. This is not likely the situation since a larger $q$-range has to be taken into consideration when calculating $|F(q)|^2$ in order to recover the intensity. Secondly, as disorder of both kinds increase intensity are removed from the peaks into the diffuse background. This causes an increasing underestimation of $F(q)$ with increasing diffraction order, since the integration is only performed over the peaks. Worthington
2.3. CAillé THEORY (CT)

and McIntosh (1974) have suggested to integrate each peak from $q = q_h - q_1/2$ to $q = q_h + q_1/2$ to recover the lost intensity. Thus, for SANS this does unfortunately not remove the problem, instead it changes the problem to a problem of separating the diffuse background from the incoherent scattering. This is not the situation for SAXS under normal conditions, since the incoherent scattering can be neglected. Finally depending of the phase, only 3-5 orders of diffraction peaks can be observed (McIntosh and Simon, 1986) due to the increasing width of the peaks. This results in a quite low resolution of the scattering length density profile calculated from Eq. (2.15). It is possible to overcome the problems with the form factors and the removed intensity by focusing on the $q$-range close to the two peaks and try to fit them to a structure factor, which adequately describes the system fluctuations. Based on Eq. (2.14) it is then possible to expand the Fourier method by calculating the form factors from an integration of the structure factor’s fitted expression.

2.3 Caillé Theory (CT)

Caillé has developed a theory for structure factors of smectic A liquid crystals (Caillé, 1972). The theory is based on de Gennes (1969) simple harmonic description of free energy density, $g$. By introducing the coarse-grained displacement variable, $u(\vec{r}, z)$, de Gennes obtained the following expression,

$$g = \frac{1}{2} K (\nabla^2 \perp u)^2 + \frac{1}{2} B \left( \frac{\partial u}{\partial z} \right)^2, \quad (2.22)$$

where $K$ is the layer bending modulus and $B$ is the bulk modulus of compression. As it can be seen the variations of $u$ in the $\vec{r}$-direction parallel to the bilayer plane are taken into account in the description, which is in contrast to the discrete one-dimensional description of the layer fluctuations, given by Eq. (2.16) and Eq. (2.17).

Helfrich (Helfrich, 1978) applied the harmonic description from Eq. (2.22) to multi-lamellar lipid bilayers in the fluid $L_\alpha$-phase. But it was first later Leibler and Lipowsky (Leibler and Lipowsky, 1987) actually showed it is valid to apply Eq. (2.22) to the description of stacks of unbound fluid lipid bilayers. The description breaks however down when an outer osmotic pressure holds the bilayers together (the bilayers are bound). Because as a consequence of applying Eq. (2.22), it is simply impossible to add up all the microscopic and steric forces acting between the bilayers in a mean-field-like way (Lipowsky and Leibler, 1986).

Assuming the bilayer stack consists of an infinite number of layers and applying the equipartition theorem to the Fourier components of the free energy density in Eq. (2.22), Caillé obtained

$$\langle (u(\vec{r}, z) - u(0, 0))^2 \rangle = \frac{k_B T}{4\pi\sqrt{K B}} \left[ 2\gamma + 2 \log \left( \frac{\pi r}{a} \right) + E_1 \left( \frac{r^2}{4z\sqrt{K/B}} \right) \right], \quad (2.23)$$

which is valid for $z \gg a^2/\sqrt{K/B}$. $a$ is a low-limit cut-off corresponding to molecular
distances, $\gamma$ is Euler’s constant and $E_1(x)$ the exponential integral function.

$$E_1(x) = \int_x^\infty \frac{e^{-t}}{t} \, dt. \tag{2.24}$$

Assuming the fluctuations $u(\vec{r}, z)$ are Gaussian as in Eq. (2.17), Caillé derived an expression for the structure factor close to the $h$’th diffraction peak, by inserting Eq. 2.23 in Eq. (2.5)

$$S(0, 0, q_z) = \langle s(0, 0, q_z) \rangle \propto \left( q_z - \frac{2\pi h}{d} \right)^{-2+\eta_h}, \tag{2.25}$$

where

$$\eta_h = \frac{\pi}{2d^2} \frac{k_B T}{\sqrt{KB}} h^2. \tag{2.26}$$

$B$ depends in practice on the layer thickness, $d_B$. $\eta_h$ can be corrected for the finite-size by applying the finite-size form of $B$ (Helfrich, 1978) to Eq. (2.26), and is given by

$$\eta_h(d_B/d) = \eta_h \cdot (1 - d_B/d)^2. \tag{2.27}$$

Als-Nielsen et al. (Als-Nielsen et al., 1980) and Safinya et al. (Safinya et al., 1986) have experimentally verified the general power-law behavior in Eqs. (2.25) and (2.26) for smectic A liquid crystal systems and the lamellar $L_\alpha$-phase of quaternary microemulsion systems respectively. Safinya et al. confirmed in the same work the finite-size form, Eq. (2.27), by a systematic experimental study of the system in a wide range of inter-bilayer distances.

Wack and Webb (Wack and Webb, 1989a) have managed to fit scattering from partially, but close to fully, hydrated oriented di-acyl-phosphatidylcholine bilayers in the $L_\alpha$-phase to Eq. (2.25) in a narrow interval around $q_h$, even though they do not use the exponents which are in accordance with Eq. (2.26). Power-law behavior has also been observed in similar experimental systems by Smith et al. (Smith et al., 1987), but without any further analysis of the quantitative relation to Eq. (2.26).

By considering a cylindrical stack of $N+1$ nearly flat bilayers with radius, $R$, Zhang et al. have been able to modify the theory so it accounts for the the finite size of the bilayer stack (Zhang et al., 1994). For small values of $r$, $r^2 < 4z\sqrt{K/B}$, Zhang et al. obtained

$$\langle (u(\vec{r}, z) - u(0, 0))^2 \rangle = \frac{k_B T}{4\pi \sqrt{KB}} \left[ \sum_{k=1}^{N} \frac{1 - \cos(k \pi z / Nd)}{k} + \frac{r^2}{4z \sqrt{KB}} \right], \tag{2.28}$$

$$\simeq \frac{k_B T}{4\pi \sqrt{KB}} \left[ \gamma + \log \left( \frac{\pi z}{d} \right) + \frac{r^2}{4z \sqrt{KB}} \right], \tag{2.29}$$

where the last step is only valid in the limit of large $N$. By applying Eq. (2.29) to Eq. (2.5) and perform the powder-averaging according to Eq. (2.7) one gets an one-dimensional expression similar to Eq. (2.9) in the limit $qR \gg 1$, with

$$\langle (u_k - u_0)^2 \rangle_{CT} = \frac{d^2}{2\pi^2 \eta_h [\gamma + \log(\pi k)]} \sim \log(k) \text{ for large } k, \tag{2.30}$$

$$S_{1,CT}(q) = N + 2 \sum_{k=1}^{N} (N - k) \cos(kq) e^{-\left(\frac{d}{\pi} \right)^2 q^2 \eta_1 \gamma (\pi k)^{-1} (\frac{d}{\pi})^2 q^2 \eta_1}, \tag{2.31}$$
where CT denotes "Caillé Theory". The expression is similar to the expression obtained by Nallet et al. (Nallet et al., 1993). From a numerical study of Eq. (2.31), it can also be seen that the power-law behavior is preserved, since for the \( h \)’th peak the limit
\[
|q - q_h| \gg 1/Nd
\]
gives
\[
S_{1,\text{CT}}(q) \propto \left( q - \frac{2\pi h}{d} \right)^{-1+\eta_h}.
\]  
(2.32)
A comparison of Eqs. (2.30) and (2.31) with Eqs. (2.18)-(2.21) shows that Caillé theory mathematically includes disorder aspects between disorder of the first and second kind, since the fluctuations in position only exhibit a logarithmic dependency on the number of layers, \( k \). Drawing a physical parallel, the Caillé theory includes disorder aspects of the both first and second kind. The term Caillé theory is often exclusively connected to the asymptotic power-law behavior described by Eqs. (2.25) and (2.32). In present ph.d. thesis the term denotes the full theory, valid for the whole \( q \)-range.

Multi-lamellar lipid bilayers of DPPC in the \( L_\alpha \)-phase at \( T = 50^\circ C \) have been studied by Nagle et al. in a high resolution x-ray study (Nagle et al., 1996). Based on their observations Nagle et al. managed to fit 3(4) diffraction peaks to an equation very similar to the one given by Zhang et al. (Zhang et al., 1994). Nagle et al. furthermore managed to construct low-resolution electron density profiles based on the 3(4) form factors. These form factors are obtained by integrating the fitted expression for the structure factor over the observed 3(4) diffraction peaks. The method is refereed to as Caillé theory and Fourier analysis. A simple integration of the peaks, which is a usual way of determining the form factors, are also done by Nagle et al., but this cause major errors according to Eq. (2.14).

The same data set has also been fitted to a paracrystalline structure factor similar to Eq. (2.21). In this study Zhang et al. (Zhang et al., 1996) conclude that the Caillé structure factor, \( S_{1,\text{CT}}(q) \), fits the diffraction peaks better than the paracrystalline structure factor, \( S_{1,\text{d2}}(q) \), for the \( L_\alpha \)-phase of DPPC at \( T = 50^\circ C \).

An additional diffuse scattering contribution has been discovered by Pabst et al. (Pabst et al., 2000), but it showed neither short-range nor (quasi) long-range order. Its origin is attributed to bilayers with strong lattice defect or unilamellar vesicles, and contributes to an additional term in the intensity function Eq. (2.12). The intensity function can according to Eq. 2.12 be described as
\[
I_1(q) = |F(q)|^2 S_1(q) + N_{\text{diff}}|F(q)|^2.
\]  
(2.33)
where \( N_{\text{diff}} \) is the scale factor for the diffuse scattering from uncorrelated bilayers.

Caillé theory is principally limited to a one-dimensional structural description, since it does not take in-plan orders into account and do therefore not adequately describe the properties of the \( L_\beta' \) and \( P_\beta' \) gel phases. However, if in-plan orders are left out of consideration the bilayers are expected to be more rigid and more ordered in the stacks for the smectic H phase when compared to the smectic A phase - the "fluid" phase. In terms of Bending modulus, \( K \), and bulk modulus, \( B \), the structural data shows larger values in the \( L_\beta' \) and \( P_\beta' \)-phases than the \( L_\alpha \)-phase. It should also be noticed that fully hydrated di-acyl-phosphatidylcholines form systems of bound bilayers, which is the reason why the harmonic description in Eq. (2.22) breaks down in the smectic A phase.
Furthermore there is no evidence of decoupling between fluctuations within the bilayer and fluctuations in the bilayer position for a system of bound bilayers. A decoupling is even less likely in the Vincent of a phase transition due to significant changes in the bilayer structure and the water spacing concomitantly. Therefore the Debye approximation (Eq. (2.11)), which is a simple decoupling of the form and structure factors, might not be appropriate for fully hydrated multi-lamellar bilayer systems. Finally, interpreting scattering data in the region close to the main-phase-transition on the basis of the harmonic theory is expected to be particularly problematic. Thus, strong out-of-plane undulations starts occurring when bending rigidity decreases, which brings the system close to a critical unbinding transition (Lipowsky and Leibler, 1986; Lipowsky, 1988; Lemnich et al., 1995) due to enhancement of the interlamellar entropic repulsion forces (Helfrich, 1978). Any interpretation of the diffraction peak profiles in terms of elastic moduli of the layered system also seems obscure, since the elastic modes in the system become strongly correlated when the system comes close to the main-phase-transition.

2.4 Form factor and bilayer model

The scattering length density can, as already mentioned, be calculated from the form factors in Eqs. (2.4) and (2.15) and is proportional to the electron density for x-ray scattering. It is also worth remembering that in previous works the Fourier method has been used in calculating the scattering length density profile, obtained on di-acyl-phosphatidycholine.

The electron density profile calculated from experiments shows two peaks, identified as the electron dense head groups. A trough has also been identified as the electron-sparse methyl-terminus (Janiak et al., 1979; McIntosh and Simon, 1986). The distance between the two head groups, \( d_{pp} \), has been used to measure the response of the bilayer/water thickness to e.g. changes in the osmotic pressure (McIntosh and Simon, 1986) and/or the temperature (Chen et al., 1997). By using the difference in the scattering length density of hydrogen and deuterium Büldt et al. and Zaccai et al. have been able to obtain further structural information from neutron scattering experiments by deuterating different methyl positions in the acyl-chains, and use the scattering length density profiles to identify these (Büldt et al., 1979; Zaccai et al., 1979). A combination of neutron and x-ray can also be used to obtain independent information from small-angle scattering data (Wiener and White, 1991a,b; Klose et al., 1996). Fitting a parameterized description of the scattering length density profile to a calculated scattering length density profiles is another way to obtain further structural information from small-angle neutron and x-ray scattering data (King and White, 1986; Wiener et al., 1989; White and Wiener, 1995). The parameterization describes either different parts of the bilayers as strips with constant scattering length density or the position and spatial extension of specific atomic groups.

Assuming bilayers consist of non-fluctuating strips Nallet et al. derived a simple expression for \( |F(q)|^2 \), based on Eq. (2.12) together with Eq. (2.31) and corrected to three dimensions. With the obtained expression Nallet et al. (1993) became able to fit whole data-set of SANS and SAXS scattering data of charged surfactant bilayers-
2.4. FORM FACTOR AND BILAYER MODEL

/water lamellar systems, and not only the neighborhood of the diffraction peaks. The obtained expression is written as,

\[ I(q) = \frac{|F(q)|^2 S_C(q)}{q^2}. \] (2.34)

With the obtained expression Nallet et al. (Nallet et al., 1993) were able to fit not only the neighborhood of the diffraction peaks, but the whole data set.

Lemmich et al. (Lemmich et al., 1996) have used a similar approach to write down an expression for the form factor for neutron scattering data. They modelled the bilayer as consisting of four Gaussian fluctuating strips with constant and distinct scattering length densities. The strips, representing the two head group regions (\(\rho_H\)), the acyl-chains layer (\(\rho_L\)), and the water layer (\(\rho_W\)), where the \(\rho\)'s are the different scattering length densities, are illustrated in Figure 2.2. The acyl-oxy groups are a

![Figure 2.2: In order to determine the scattering length density profile of the bilayer, different parameterized models are used. 3GSM (3 Gaussian Strip Model) and 4GSM (4 Gaussian Strip Model) are strip models consisting of independent Gaussian fluctuating strips with distinct and different scattering length densities. 3GSM consists of three strips describing the two head groups, the two half lipid acyl-chain thickness and the water layer. The two head groups and two half lipid tails have equal scattering length density in pairs. In the case of 4GSM two additional strips are used to describe the methyl-terminus in the middle of the bilayer. GM (Gaussian Model) is a Gaussian description of the scattering length density profile, and it consists of two Gaussian distribution functions; one describing the head groups and one describing the methyl-terminus.

part of the acyl-chains layer, since the acyl-chains are fully deuterated and the size of the scattering lengths for deuterium, carbon and oxygen are comparable and somewhat larger than the scattering length density for hydrogen (Sears, 1986). By splitting the strip describing the hydrophobic core into two independently strips the model becomes symmetric around the middle of the bilayer. The thickness of the layers are assumed to fluctuate independent with a Gaussian distribution around the mean values \(d_H\), \(d_L\) (the half of the hydrophobic part), and \(d_W\) with the corresponding standard deviations \(\sigma_H\), \(\sigma_L\), and \(\sigma_W\). This profile model is denoted the 3 Gaussian Strip Model or 3GSM.
and applying it to Eq. (2.3) the following form factor can be obtained,

\[
f(q) = \rho_H \int_0^{d_H} e^{-iqz} dz + \rho_L \int_{d_H}^{d_H+2d_L} e^{-iqz} dz + \rho_M \int_{d_H+2d_L}^{2(d_H+d_L)} e^{-iqz} dz
\]

\[
= \frac{1}{iq} \left[ \rho_H(1 - e^{-2iq(d_H+d_L)}) + (\rho_L - \rho_H)e^{-iqd_H}(1 - e^{-2iqd_L}) \right]
\] (2.35)

The form factor uses the scattering length density for water, \( \rho_W \), as reference. By combining the model with the one-dimensional intensity function in Eq. (2.11), the Caillé structure factor in Eq. (2.31) and correct it to the three dimensions in Eq. (2.9) Lemmich et al. (Lemmich et al., 1997) obtained

\[
I_{SCT}(q) = \frac{|F(q)|^2 S_{1,CT} + N(|f(q)|^2) - |F(q)|^2}{q^2}
\] (2.36)

where SCT denotes Special Caillé Theory. \( F(q) \) and \( |f(q)|^2 \) in the 3 Gaussian Strip Model are then given by

\[
F(q) = |\langle f(q) \rangle| = \frac{2\rho_H}{q} \left\{ \sin [(d_H + d_L)q] e^{-\frac{1}{2}(\sigma_H^2 + \sigma_L^2)} + (\bar{\rho} - 1) \sin [d_Lq] e^{-\frac{1}{2}\sigma_L^2} \right\}
\] (2.37)

and

\[
|\langle f(q) \rangle|^2 = \frac{2\rho_H^2}{q^2} \Re \left[(1 - F_H^2F_L^2) + (\bar{\rho} - 1)^2(1 - F_L^2) + 2(\bar{\rho} - 1)(F_H(1 - F_L^2))\right].
\] (2.38)

where

\[
\bar{\rho} = \frac{\rho_L - \rho_W}{\rho_H - \rho_W}
\] (2.39)

is the reduced scattering length density and

\[
F_\nu(q) = e^{(-iqd_L - \frac{1}{2}q^2\sigma_L^2)}
\] (2.40)

is the Fourier transformation of the Gaussian distribution function, with \( \nu = H, L, W, \) and \( \Re \) denoting the real value of the complex function.

Analysis of x-ray scattering data shows that the electron density in the middle of the bilayer has a trough of charge (Janiak et al., 1979; McIntosh and Simon, 1986), which can be ascribed to the methyl-terminus of acyl-chain. The 3GSM model does not take this into account, which is why we have made an expansion of the strip model, denoted the 4 Gaussian Strip Model or 4GSM. The 4GSM model takes the methyl-terminus into account and has the form factor given as,

\[
f(q) = \rho_H \int_0^{d_H} e^{-iqz} dz + \rho_L \int_{d_H}^{d_H+d_L} e^{-iqz} dz + \rho_M \int_{d_H+d_L}^{d_H+2d_L+2d_M} e^{-iqz} dz
\]

\[
+ \rho_L \int_{d_H+d_L+2d_M}^{2(d_H+d_L+2d_M)} e^{-iqz} dz + \rho_H \int_{2(d_H+d_L+2d_M)}^{2(d_H+2d_L+2d_M)} e^{-iqz} dz
\]

\[
= \frac{1}{iq} \left[ \rho_H(1 - e^{-2iq(d_H+d_L+d_M)}) + (\rho_L - \rho_H)e^{-iqd_H}(1 - e^{-2iq(d_L+d_M)})
\]

\[
+ (\rho_M - \rho_L)e^{-iqd_L}(e^{-iqd_L} - e^{-iq(d_L+2d_M)}) \right]
\] (2.41)
where $\rho_W$ again is used as reference. $F(q)$ and $\langle |f(q)|^2 \rangle$ are then given as

$$F(q) = |\langle f(q) \rangle| = \frac{2\tilde{\rho}_H}{q} \left\{ (\tilde{\rho}_M - \tilde{\rho}_L) \sin |d_M q| e^{-\frac{1}{2}\sigma^2_H q^2} + (\tilde{\rho}_L - \tilde{\rho}_L) \sin [(d_L + d_M) q] e^{-\frac{1}{2}(\sigma^2_L + \sigma^2_M) q^2} + \sin [(d_H + d_L + d_M) q] e^{-\frac{1}{2}(\sigma^2_L + \sigma^2_M) q^2} \right\}$$

and

$$\langle |f(q)|^2 \rangle = \frac{2\tilde{\rho}_H^2}{q^2} R[ (\tilde{\rho}_M - \tilde{\rho}_L)^2 (1 - F^2_M + F_L (F^2_M - 1)) + (\tilde{\rho}_L - 1)^2 (1 - F_L) (1 + F_L F^2_M) + (1 - \tilde{\rho}_M) (F_H - F_L F^2_M) + 2(\tilde{\rho}_L - 1) (F_H - F_L F^2_M) + (1 - F_H)^2 F^2_L F^2_M )]$$

(2.43)

where

$$\tilde{\rho}_L = \frac{\rho_L - \rho_W}{\rho_H - \rho_W}, \quad \tilde{\rho}_M = \frac{\rho_M - \rho_W}{\rho_H - \rho_W}$$

(2.44)

and $F$ given by Eq. (2.40) with $\nu = H, L, M, W$.

A general problem with the strip models are the discontinuous step in the scattering length density between the strips. Therefore other models, describing the scattering length density of the bilayer in a continuous way, have been suggested. Wiener et al. (1989) have e.g. described the electron density of the bilayer as a summation of two Gaussians, representing the polar head groups and the methyl-terminus respectively,

$$\rho(z) = \rho_{CH_2} + \tilde{\rho}_h \left[ e^{-\frac{(z-\tilde{z}_h)^2}{2\sigma_h^2}} + e^{-\frac{(z+\tilde{z}_h)^2}{2\sigma_h^2}} \right] + \tilde{\rho}_c e^{-\frac{z^2}{2\sigma_c^2}}.$$  

(2.45)

where the electron density of the head group, $\tilde{\rho}_h$, and the methyl-terminus $\tilde{\rho}_c$ are defined relative to the methylene electron density, $\rho_{CH_2}$

$$\tilde{\rho}_h \equiv \rho_h - \rho_{CH_2}$$

(2.46)

$$\tilde{\rho}_c \equiv \rho_c - \rho_{CH_2}$$

(2.47)

The form factor can then be analytically calculated by applying Eq. 2.3 to Eq. 2.45

$$\langle f(q) \rangle = F(q) = 2F_h(q) + F_c(q)$$

(2.48)

$$F_i(q) = \sqrt{2\pi\sigma_i^2} \tilde{\rho}_i e^{-\frac{q^2 \sigma_i^2}{2}} \cos(qz_i)$$

(2.49)

where $i$ denotes $h$ and $c$ for the head groups and the methyl-terminus respectively (note $z_c = 0$), and $\sigma_i$ is the standard deviation giving the widths of the respectively parts of the bilayer. By using the form factor from Eq. (2.49) in the intensity function (cf. Eq. (2.33)) with the Caillé structure factor, $S_{1,CT}(q)$, in Eq. (2.31) and correct it to the three dimensions as in Eq. (2.9), Pabst et al. obtained,

$$I_{MCT}(q) = \frac{|F(q)|^2 S_{1,CT}(q)}{q^2} + N_{d_{eff}} |F(q)|^2.$$  

(2.50)

where MCT denotes Modified Caillé Theory. With this expression Pabst et al. (Pabst et al., 2000) managed to fit their whole data set.
2.5 Paracrystalline Theory (PT)

When modelling scattering data obtained from multi-lamellar bilayers of di-acyl-phosphatidylcholines in the $L_{\beta'}$ and $L_{\alpha}$-phases, there are several reasons why the Caillé theory can not be expected to be an ideal theory. First of all the theory only works for smectic A phases. Which is why the finite in-plane shear modulus has to be taken into account in the smectic H phases, e.g. the "gel" phases $L_{\beta'}$ and $P_{\beta'}$, and makes Eq. (2.22) inadequate.

Because of the absence of a complete Hamiltonian theory including all aspects of the fluctuating system, Lemmich et al. (Lemmich et al., 1996) have derived a simple geometric model by combining the 3 Gaussian Strip Model (3GSM) with a paracrystalline structure factor, comprising disorder of the second kind. Lemmich et al. did not decouple the form- and structure factors according to the Debye approximation Eq. (2.11), but choose the more accurate expression in Eq. (2.10). The one-dimensional paracrystalline theory given by Hosemann and Bagchi (Hosemann and Bagchi, 1962b) makes the basis of this theory together with the assumption that each of the $N$ repeat units in the stack of the bilayers can be described as layers with distinct and different scattering length densities. The fluctuation of the thickness for each of the layers are assumed to be independent and follow a Gaussian distribution. Based on these assumptions the form factor can be calculated from Eq. (2.3), and by applying Eqs. (2.9) and (2.10) one obtains the geometric model (Lemmich et al., 1996).

\[ I_{PT}(q) = \frac{\Gamma_{PT}}{q^4} \left[ i_B(q) + \frac{1}{N} i_C(q) \right] \]  

(2.51)

with

\[
\begin{align*}
  i_B(q) &= \Re \left[ \frac{(1 - F_W)(1 - F_H^2 F_L^2) + (\tilde{\rho} - 1)^2 (1 - F_L^2)(1 - F_H^2 F_W)}{1 - F_D} \right] \\
  &\quad + \Re \left[ \frac{2(\tilde{\rho} - 1) F_H (1 - F_W)(1 - F_L^2)}{1 - F_D} \right] \\
  i_C(q) &= \Re \left[ F_W (1 - F_D^N) \left( \frac{(1 - F_H^2 F_L^2) + (\tilde{\rho} - 1) F_H (1 - F_L^2)}{1 - F_D} \right)^2 \right],
\end{align*}
\]

(2.52)

where $\tilde{\rho}$ is given by Eq. (2.39) and $F_\nu(q)$ by Eq. (2.40) with $\nu = H, L, W, D$ (where $d_D = d$), i.e. $F_D = F_H^2 F_L^2 F_W$. $\Re$ denotes the real value of the function, the subscript PT stands for 'Paracrystalline Theory' and $\Gamma_{PT}$ is a normalization constant. A numerical study shows that Eq. (2.51) in the high-$q$ limit takes the form $I(q \to \infty) \sim q^{-4}$.

The paracrystalline theory described in Eqs. (2.51) and (2.52) takes however not the methyl-terminus of the acyl-chain in the middle of the bilayer into account. Thus,
it can be corrected by doing the same calculation with the 4 Gaussian Strip Model,

\[
\begin{align*}
    i_B(q) &= \Re \left\{ \frac{(\tilde{\rho}_M - \tilde{\rho}_L)^2(1 - F_L)(1 - F_M^2)(1 + F_H^2F_LF_W)}{(1 - F_{D'})} \\
    &\quad + \frac{(\tilde{\rho}_L - 1)^2(1 - F_L)(1 + F_LF_M^2)(1 - F_H^2F_W)}{(1 - F_{D'})} \\
    &\quad + \frac{(1 - \tilde{\rho}_M)^2F_L(1 - F_M)(1 + F_M)(1 - F_H^2F_W)}{(1 - F_{D'})} \\
    &\quad + \frac{2(\tilde{\rho}_M - \tilde{\rho}_L)F_HF_L(1 - F_M^2)(1 - F_W)}{(1 - F_{D'})} \\
    &\quad + \frac{2(\tilde{\rho}_L - 1)F_H(1 - F_L^2F_M^2)(1 - F_W) + (1 - F_H^2F_L^2F_M^2)(1 - F_W)}{(1 - F_{D'})} \right\}
\end{align*}
\]

\[
i_C(q) = \Re \left[ F_W(1 - F_{D'}^N) \left( \frac{(1 - F_H)(1 + F_HF_LF_M^2) + \tilde{\rho}_M F_HF_L(1 - F_M^2)}{1 - F_{D'}^2} \right) \right.
\]

\[
\left. + \frac{\tilde{\rho}_L F_H(1 - F_L)(1 + F_LF_M^2)}{1 - F_{D'}^2} \right)^2
\]

where \(\tilde{\rho}_\nu\) is given by Eq. (2.44) with \(\nu = L\) and \(M\), and \(F\) given by Eq. (2.40) with \(\nu = H, L, M, W, D\) i.e. \(F_{D'} = F_H^2F_L^2F_M^2F_W\). To discriminate the two versions of paracrystalline theory, 3GSM and 4GSM will be denoted PT (3GSM) and PT (4GSM) respectively.

So far an important part of the discussion has regarded the acceptance of separating the form and structure factors, which is an important aspect when distinguishing between paracrystalline theory, Eq. (2.51), and Caillé theory, Eq. (2.36) and Eq. (2.50). It can therefore be very instructive to perform an analysis of the scattering data in terms of Decoupled Paracrystalline Theory (DPT), where the \(form\) and \(structure\) factors are decoupled

\[
I_{DPT}(q) = \frac{|F(q)|^2S_1d_2 + N(|\langle f(q) \rangle|^2) - |F(q)|^2}{q^2}
\]

The last term in the numerator of Eqs. (2.36) and (2.54) is very important, since it includes the fluctuation in the form factor description, which is in contrast to Nallet et al. (Nallet et al., 1993). The distribution functions of the layers are the primary result from fitting scattering data to either Eq. (2.51), Eq. (2.36), or Eq. (2.54), wherefrom the structural parameters, \(d_H, d_W, d_L, \sigma_H, \sigma_W, \) and \(\sigma_L\), can be extracted.

Another valuable structural parameter of the lipid bilayer, which can be obtained from the results of the fits, is the average interfacial area per lipid molecule, which can be calculated from the volume of the lipid molecule, \(V_L\), through the relation

\[
A d_B = 2V_L
\]

or according to (Nagle and Tristram-Nagle, 2000)

\[
A = (V_L - V_H)/d_{C/2}
\]
where, $V_H$, is the volume of the head group and $d_{C/2}$ is half the thickness of the hydrophobic core or the effective acyl-chain length. $V_L$ can be precisely determined from volumetric measurements. For DMPC and DPPC, $V_H$ has been determined by Sun et al. (1994) to $319 \, \text{Å}^3$.

The average interfacial area per lipid molecule, $A$, can then be used to calculate the number of water molecules per lipid molecule (Nagle and Tristram-Nagle, 2000)

$$d \, A = 2(V_L - n_W V_W) \tag{2.57}$$

Finally, the modulated (rippled) bilayer surface, which is a special feature of the $P_{3r}$-phase, should be discussed. From wide-angle x-ray studies it has been revealed that at low hydration the ripples of di-acyl-phosphatidylcholine bilayers are asymmetric and form an oblique unit cell perpendicular to the bilayer plane (Sirota et al., 1988; Katsaras and Raghunathan, 1995). For fully hydrated di-acyl-phosphatidylcholine the typical ripple wavelength has been reported as app. 120-140 Å (Janiak et al., 1976; Matuoka et al., 1990), while the wavelength seems to increase at lower hydrations (Janiak et al., 1976; Wack and Webb, 1989b). For fully hydrated di-acyl-phosphatidylcholine bilayers no studies have so far reached a consensus for the ripple structure, e.g. the amplitude of the ripples, and the present ph.d. thesis will not discuss this subject any further.

The main problem with the ripples are the additional scattering peaks as described by Tardieu et al. (Tardieu et al., 1973). Firstly, a (0,1) peak characterizing the ripple repeat distance can be observed, while higher order peaks such as (0,2), (1,1) and (2,1) appear as “shoulders” on the main bilayer diffraction peaks. At low hydration the intensity of these additional peaks is quite high, and decreases with increasing hydration (Wack and Webb, 1989b), while the (0,1) and ($n,1$) peaks still can be detected in fully hydrated bilayer systems (Matuoka et al., 1990).
Chapter 3

Analysis of Data and Comparison of Methods

The quality of the fits are determined from the minimum of $\chi^2_{\text{reduced}}$, which is given by

$$
\chi^2_{\text{reduced}} = \frac{1}{N_{\text{free}}} \sum \left( \frac{I_{\text{obs}} - I_{\text{cal}}}{\sigma_{\text{obs}}} \right)^2
$$

(3.1)

where $N_{\text{free}} = N_{\text{data points}} - N_{\text{fit parameter}} - 1$ is the number of degree of freedom, $N_{\text{data points}}$ is the number of data points and $N_{\text{fit parameter}}$ is the number of fit parameters. It should be noticed that for an optimal fit to any data set $\chi^2_{\text{reduced}}$ will be approximately equal to one, provided the errors are normally distributed. In practice, the value of $\chi^2_{\text{reduced}}$ will be larger than one for experimental data.

The analysis of small-angle scattering data is based on paracrystalline theory Eq. (2.51), decoupled paracrystalline theory Eq. (2.54) and two versions of the Caillé theory; special Caillé theory Eq. (2.36) and modified Caillé theory Eq. (2.50) and will be used with different models of the scattering length density profile.

3.1 Analysis of Small-Angle Neutron Scattering Data (SANS)

The analysis of SANS is based on small-angle neutron scattering data obtained on multi-lamellar samples of DMPC-d$_{54}$ and DPPC-d$_{62}$ by Kell Morensen and Jesper Lemmich at Risø National Laboratory. The lipid samples are dissolved in a D$_2$O buffer [50 mM Hepes ∼pD 7.2, 10 mM NaCl, 1 mM NaN$_3$, and 60 µM ethylenediamine tetraacetic acid (EDTA)] to a concentration of ∼15 wt% lipid (Lemmich et al., 1996) and obtained at temperatures $T = 32.0^\circ$C for DMPC-d$_{54}$ and $T = 48.8^\circ$C for DPPC-d$_{62}$, corresponding to the $L_\alpha$-phase. In order to cover a large interval in $q$-space two different instrumental set-ups have been used. With a sample-to-detector distance of 3 m and 9 Å neutrons the covered $q$-range is 0.007-0.06 Å$^{-1}$ and it changes to 0.07-0.58 Å$^{-1}$ for a sample-to-detector distance of 1 m and 2.8 Å neutrons.
3.1.1 Instrumental Resolution and Deconvolution of SANS Scattering Data

A nonlinear least-square fitting routine has been used in the analysis of small-angle neutron scattering data, and the smearing, caused by the different instrument geometric set-ups, has been taken into account as described by Pedersen et al. (Pedersen et al., 1990).

The used instrumental set-up has a wavelength spread of $\Delta \lambda/\lambda = 0.18$, which, despite the large difference in $\lambda$, still makes it possible to elucidate the features of the $(n,0)$ peak shapes in SANS (Figure 3.1).

![Figure 3.1](image)

Figure 3.1: The figure shows the principle of deconvolution. The solid line is the fitted function convoluted with the resolution function, described by Pedersen et al., while the dotted line shows the function without being convoluted with the resolution function. The parameters are obtained from a fit performed on a DMPC-$d_{54}$ SANS spectrum.

3.1.2 Accuracy of SANS Scattering Data

For all SANS data three orders of diffraction peaks have been observed. The $(1,0)$, $(2,0)$ and $(3,0)$ peaks are illustrated in Figure 3.2. From the figure it can also be seen that the shape of the diffraction peaks are Gaussian, which might be caused by the smearing since the wavelength spread and instrumental set-ups cause a Gaussian convolution of the scattering data. The $(0,1)$ reflection of the ripples in the $P_{\beta'}$-phase is furthermore shown as a very broad peak between $q \simeq 0.04 \, \text{Å}^{-1}$ and $q \simeq 0.06 \, \text{Å}^{-1}$ for both DMPC-$d_{54}$ and DPPC-$d_{62}$, while higher order reflections ($(0,n)$ $n > 1$) are not detected in any of the scattering data. The SANS scattering data also shows an increasing width of the diffraction peaks as the diffraction order increases. This reflects the quasi-long range order of the multi-lamellar lipid bilayer system, which is characteristic for liquid crystals.
3.1. Analysis of Small-Angle Neutron Scattering Data (SANS)

3.1.3 Paracrystalline Theory

The analysis of small-angle neutron scattering data based on paracrystalline theory are in the present Ph.D. thesis used together with two different models of the scattering length density profile: 3GSM and 4GSM.

**Fit parameter set**

In PT (3GSM) three different types of layers have been taken into account, the head group, the acyl chain and the water layer (cf. Section 2.5 Eq. (2.51) with \( i_B \) and \( i_C \) given by Eq. (2.52)). The same layers have been taken into account in PT (4GSM); though an additional methyl-terminus layer in the middle of the bilayer has been applied (\( i_B \) and \( i_C \) in Eq. (2.52) are replaced with Eq. (2.53)).

Fits performed with PT (3GSM) require 8 different structural parameters, \( \tilde{\rho} \), \( N \), \( d_H \), \( d_L \), \( d_W \), \( \sigma_H \), \( \sigma_L \), \( \sigma_W \), together with a scale factor, \( \Gamma \), and a background value, \( I_{\text{bac}} \), for each SANS spectra. PT (4GSM) has correspondingly 11 structural parameters, \( \tilde{\rho}_L \), \( \tilde{\rho}_M \), \( N \), \( d_H \), \( d_L \), \( d_W \), \( \sigma_H \), \( \sigma_L \), \( \sigma_M \), \( \sigma_W \).

In order to obtain reliable fits, the ratio between number of data points and number of free parameters should be as large as possible. We have however only a small number of data points available, which is why the ratio between number of data points and number of free parameters only can be increased through a reduction in the number of free parameters. In the present Ph.D. thesis I have chosen to let all widths of the layer thickness distribution have the same value relative to the different layers for PT (3GSM). Thus, \( \sigma_\nu = \sigma_{\text{relative}} \cdot d_\nu \) with \( \nu \) denoting the individual layers, \( d_\nu \) the individual thickness of the layers and \( \sigma_{\text{relative}} \) being the only fitted layer thickness distribution parameter. By doing this the number of free structural parameters in the scattering function can be reduced from 8 to 6, consisting of \( \tilde{\rho} \), \( \rho_L \), \( \rho_M \), \( d_H \), \( d_L \), \( d_W \), \( \sigma_{\text{relative}} \), a scale factor, \( \Gamma \), and a background value, \( I_{\text{bac}} \), for each SANS spectra used in the fit with PT (3GSM). The same procedure is possible with PT (4GSM), thus here the fitted number of free structural parameters decreases to 8, consisting of \( \tilde{\rho}_L \), \( \rho_M \), \( N \), \( d_H \), \( d_L \), \( d_W \), \( \sigma_{\text{relative}} \).

Applying the diffused term, \( N_{\text{diffuse}} |F(q)|^2 \), as suggested by Pabst et al. (Pabst et al., 2000) where \( F(q) \) is given either by Eq. (2.37) or Eq. (2.42) for 3GSM and 4GSM respectively, requires an extension of the structural parameter set with a scale factor for the diffuse term, \( N_{\text{diffuse}} \).

In the following the notation PT\(_r\) (3GSM) will be used to denote PT (3GSM) with relative widths of the layer thickness distribution (referring to the reduced parameter set) while PT\(_a\) (3GSM) will be used to denote PT (3GSM) with absolute widths of the layer thickness distribution (referring to the full parameter set). The notation will be applied to all sections in Chapter 3.

**Fitting procedure**

For both PT (3GSM) and PT (4GSM) two interval of \( q \)-values have been used in our fits, one at low \( q \)-values and one at high \( q \)-values. First, all structural parameters but \( N \) are fitted to the high-\( q \) spectra together with a scale and background factor until
a stable minimum are reached (Type I). Secondly, all structural parameters are fixed and \( N \) let free and fitted to the low-\( q \) spectra together with the scale and background factor until a stable minimum again is reached (Type II). Subsequently new Type I fits are performed, this time based on the \( N \) value obtained in the Type II fits and so it continues alternating between Type I and II fits until consisting fits are reached.

**Observations**

For PT\(_r\) (3GSM), where the widths are relative, none of the fits show sufficient intensity for the third order peak. For both DMPC-d\(_{54}\) and DPPC-d\(_{62}\) in the \( L_\alpha \)-phase, the fitted function slightly overestimates the scattered intensity in the tail-region of the zeroth order diffraction peak (Figure 3.2a,b), while underestimating the scattered intensity between both the zeroth and first and the first and second order diffraction peaks. By adding a diffuse term, \( N\_\text{diffuse} \), it is however possible to obtain a well reproduced scattered intensity between the first and second order diffraction peaks, whereas no significant improvement are observed for the scattered intensity in the tail-region of the zeroth order diffraction peak (Figure 3.2d,c). The extension also causes a better agreement between the fits and the third order diffraction peak, though it is still not well reproduced. The agreement between the fitted and scattered intensity in the tail-region of the zeroth order diffraction peak is not improved very much by the diffuse term.

In the \( P_\beta' \)-phase the scattering function both overestimates the tail-region of the zeroth order diffraction peak and underestimates the scattered intensity of the third order diffraction peak and the first and second order diffraction peaks (Figure 3.2e,f). An improved agreement between the scattering function and the spectra of DMPC-d\(_{54}\) and DPPC-d\(_{62}\) can however be obtained by adding the diffuse term, \( N\_\text{diffuse} \), to the scattering function (Figure 3.2h,g); though there are still some mismatch between the zeroth and the first order diffraction peaks. It can furthermore be seen that the improvement is larger for DPPC-d\(_{62}\) than for DMPC-d\(_{54}\).

None of the present models for the scattering function take the ripples in the \( P_\beta' \)-phase into account, and due to the appearance of the \((0,1)\) and \((n,1)\) peaks, this can explain the above mentioned mismatch in the tail-region of the zeroth order diffraction peak and between the first and second order diffraction peaks. A study performed by Matuoka et al. (Matuoka et al., 1990) on fully hydrated DMPC-d\(_{54}\) bilayers in the \( P_\beta' \)-phase with synchrotron x-ray at very high resolution, shows that the intensity of the \((1,1)\) peak relative to the \((1,0)\) peak is very small, whereas the \((2,1)\) peak is larger and in intensity comparable with the intensity of the \((2,0)\) peak. At low temperatures, i.e. close to the pre-transition, the intensity of the \((1,1)\) and \((2,1)\) peaks increase relative to the \((1,0)\) and \((2,0)\) peaks. The main contributions to the scattering intensity are therefore expected to come from the \((n,1)\) peaks, particularly \((1,1)\) and \((2,1)\). This means that an \( n \)'th order diffraction peak is artificially broadened and shifted towards a higher \( q \)-value, due to the \((n,1)\) peak in our spectra. For DMPC-d\(_{54}\) and DPPC-d\(_{62}\) close to the main-phase-transition, the effect on the \((1,0)\) peak is expected to be fairly small, whereas a relatively stronger effect is expected in the region close to the \((2,0)\) peak, which explains the mismatch between the scattering intensity and the fit.
3.1. ANALYSIS OF SMALL-ANGLE NEUTRON SCATTERING DATA (SANS)

Figure 3.2: The figure shows fits based on paracrystalline theory and the 3GSM model. The fits are performed with relative widths of the layer thickness distribution. The plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $L_\alpha$ with diffuse term (wd), (d) DPPC $L_\alpha$ wd, (e) DMPC $P_{\beta'}$, (f) DPPC $P_{\beta'}$, (g) DMPC $P_{\beta'}$ wd and (h) DPPC $P_{\beta'}$ wd.
Figure 3.3: The figure shows fits based on paracrystalline theory and the 3GSM model. The fits are performed with absolute widths of the layer thickness distribution. Again, the plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $L_\alpha$ with diffuse term (wd), (d) DPPC $L_\alpha$ wd, (e) DMPC $P_{\beta'}$, (f) DPPC $P_{\beta'}$, (g) DMPC $P_{\beta'}$ wd and (h) DPPC $P_{\beta'}$ wd.
In order to overcome the problem with lack of intensity in the third order diffraction peak, especially in the \( L_\alpha \)-phase, we also let all widths of the layer thickness distribution be independent (absolute) for the different layers for PT (3GSM). Thus, by substituting \( \sigma_{\text{relative}} \) with \( \sigma_H \), \( \sigma_L \) and \( \sigma_W \) respectively, we are able to perform new fits. First, the width of the layer thickness distribution for the head group, \( \sigma_H \), is set to zeroth in order to achieve a stable minimum. By doing this the scattering function fits both the third order diffraction peak in the \( L_\alpha \)-phase as well as the scattered intensity between the zero and first and the first and second order diffraction peaks (Figure 3.3). Again, a slightly better result can be obtained by adding a diffuse term, \( N_{\text{diffuse}} \), to the scattering function (Figure 3.3c,d,g and h).

The scattering function gives in general better agreements between the fits and data in both the \( L_\alpha \) and \( P_\beta' \)-phases for PT\(_a\) (3GSM) with independent (absolute) widths. The agreements between the fits and spectra are furthermore mostly improved for the \( L_\alpha \)-phase. For the \( P_\beta' \)-phase it seems that the second order diffraction peak (2,0) is shifted towards lower-\( q \) values in the fitted intensity, when compared to the scattered one. No such effect is observed for the (1,0) peak. The ripples do simply not seem to affect the position of the diffraction peaks in the different fits. The artificial broadening of the \((n,0)\) peaks do therefore imply a somewhat overestimation of the fluctuations in the system. This is indeed the case with \( \sigma_W \) and \( \sigma_L \) for both DMPC-d\(_{54}\) and DPPC-d\(_{62}\), where \( \sigma_L \) is larger in the \( P_\beta' \)-phase than in the \( L_\alpha \)-phase (Figure 3.11). Going from lipid-acyl-chain order to disorder, is clearly in contrary to our expectations.

Regarding PT (4GSM), where the methyl-terminus in the middle of the bilayer is included, it has unfortunately not been possible to achieve a stable minimum for neither relative nor absolute widths of the layer thickness distribution, which is due to a parameter correlation between the methyl-terminus thickness, \( d_M \), and the width of the acyl-chain thickness, \( d_L \). This is most likely caused by the smoothing of the boundaries between the acyl-chain and the methyl-terminus layers due to an almost identical scattering length for deuterium and carbon.

Finally, it is assumed that the head group and the water region are clearly separable in the simple geometric modelling of the form factor as implied by paracrystalline theory. This is obviously a simplification, since the hydration of the head group will tend to smooth out the boundary between the two regions. As expected, this gives rise to a rather strong coupling between \( d_H \) and \( d_W \) in the fits to SANS spectra. In practice, this means the values of \( d_H \) and \( d_W \) are quite sensitive to small changes in the fitting procedure, whereas the value of the total hydrophilic layer thickness, \( d_A = 2d_H + d_W \), is expected to be stable and display less scatter.

### 3.1.4 Decoupled Paracrystalline Theory

The analysis of small-angle scattering data based on decoupled paracrystalline theory are again used with the 3GSM and 4GSM models.
Fit parameter set

In DPT (3GSM) three layers, the head group, the acyl chain and the water layer, are taking into account as for PT (3GSM) (cf. Section 2.5 Eq. (2.54) with \(F(q)\) given by Eq. (2.37) and \(\langle|f(q)|^2\rangle\) given by Eq. (2.38)). For DPT (4GSM) the methyl-terminus layer has likewise been taken into account as described for PT (4GSM). Thus, the equations for \(F(q)\) and \(\langle|f(q)|^2\rangle\) have been replaced by Eqs. (2.42) and (2.43).

Fits performed with DPT (3GSM) require 7 different structural parameters, \(\tilde{\rho}, d_H, d_L, d_{\text{repeat}}, \sigma_H, \sigma_L, \sigma_{\text{repeat}}\) together with a scale factor, \(\Gamma\), and a background value, \(I_{\text{bac}}\), for each SANS spectra. Equivalent for DPT (4GSM); though the number of structural parameters increases to 10, \(\tilde{\rho}_L, \tilde{\rho}_M, d_H, d_L, d_M, d_{\text{repeat}}, \sigma_H, \sigma_L, \sigma_M, \sigma_{\text{repeat}}\).

Again, the number of free structural parameters have been reduced by letting the widths of the layer thickness distribution be relative to the layer thickness. Thus, \(\sigma_\nu = \sigma_{\text{relative}} \cdot d_\nu\) and \(\sigma_{\text{relative}}\) is the only fitted, where \(\nu\) denotes the individual layers and \(d_\nu\) the layers individual thickness. By doing this the number of free structural parameters in the scattering function for DPT (3GSM) can be reduced to 6, consisting of \(\tilde{\rho}, d_H, d_L, d_{\text{repeat}}, \sigma_{\text{relative}}, \sigma_{\text{repeat}}\), while the parameter set for DPT (4GSM) can be reduced to 8, consisting of \(\tilde{\rho}_L, \tilde{\rho}_M, d_H, d_L, d_M, d_{\text{repeat}}, \sigma_{\text{relative}}, \sigma_{\text{repeat}}\).

Fitting procedure

The fitting of decoupled paracrystalline theory follows overall the same procedure as paracrystalline theory. Though, with the only exception of low-\(q\) spectra, where the scala factor is the only fitted parameter, which is due to problems with the fitting of the background. Note that for decoupled paracrystalline theory the number of bilayers, \(N\), can not be fitted because of correlation between \(N\) and the scala factors for each spectra (cf. Eqs. (2.21) and (2.54)).

Observations

Since there are some similarity between the observations for decoupled paracrystalline theory and paracrystalline theory the following will focus on the differences between DPT and PT.

Regarding DPT, (3GSM), the scattering function is able to fit the third order diffraction peak for both DMPC-\(d_{54}\) and DPPC-\(d_{62}\) in the \(L_\alpha\)-phase (Figure 3.4a,b), which is in clear contrast to the results obtained with paracrystalline theory. DPT, (3GSM) also obtains a better fitted intensity in the \(L_\alpha\)-phase than PT (3GSM). Though, with the exception of the tail-region of the zeroth peak, which is better described by PT (3GSM). Adding the diffuse term, \(N_{\text{diffuse}}\), to the scattering function does not cause any significant improvements, as it does with PT (3GSM) (Figure 3.4c,d)

In the \(P_\gamma\)-phase DPT (3GSM) does not obtain the same good agreement as in the \(L_\alpha\)-phase (Figure 3.4e,f). Though, both the second and third order diffraction peaks seems to occur at lower \(q\)-values in the fitted function than in the SANS spectra, which may be due to the presence of \((n,1)\) ripple peaks. For DPPC-\(d_{62}\) in the \(P_\gamma\)-phase an addition of the diffuse term, \(N_{\text{diffuse}}\), seem to give better fits, while worse for
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![Figure 3.4: The figure shows fits based on decoupled paracrystalline theory and the 3GSM model. The fits are performed with relative widths of the layer thickness distribution function. The plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $L_\alpha$ with diffuse term (wd), (d) DPPC $L_\alpha$ wd, (e) DMPC $P_{\beta'}$, (f) DPPC $P_{\beta'}$, (g) DMPC $P_{\beta'}$ wd and (h) DPPC $P_{\beta'}$ wd.](image-url)
Figure 3.5: The figure shows fits based on decoupled paracrystalline theory and the 3GSM model. The fits are performed with absolute widths of the layer thickness distribution function. Again, the plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $L_\alpha$ with diffuse term ($w_d$), (d) DPPC $L_\alpha$ $w_d$, (e) DMPC $P_{\beta'}$, (f) DPPC $P_{\beta'}$, (g) DMPC $P_{\beta'}$ $w_d$ and (h) DPPC $P_{\beta'}$ $w_d$. 
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DMPC-d\textsubscript{54} (Figure 3.4g,h).

When the widths of the layer thickness distribution are let independent (absolute) it is not possible to fit DPT\textsubscript{a} (3GSM) to low-q spectra for both DMPC-d\textsubscript{54} and DPPC-d\textsubscript{62} in the L\textsubscript{α}-phase (Figure 3.5a,b). In general, it can furthermore be seen that fits obtained with DPT\textsubscript{a} (3GSM) are similar to the ones obtained with DPT, (3GSM). Though, with the exception of DMPC-d\textsubscript{54} in the P\textsubscript{β}-phase where the obtained fits are worse with absolute widths than relative (Figure 3.5e). For DPPC-d\textsubscript{62} in the P\textsubscript{β}-phase just the opposite is the case, since here the best fits are performed with absolute and not relative widths (Figure 3.5f).

An addition of the diffuse term, N\textsubscript{diffuse}, to DPT\textsubscript{a} (3GSM) makes it possible to fit the model to low-q spectra for both DMPC-d\textsubscript{54} and DPPC-d\textsubscript{62} in the L\textsubscript{α}-phase. As with PT\textsubscript{a} (3GSM) the diffuse term also causes slightly better fits for DPT\textsubscript{a} (3GSM) in both the L\textsubscript{α} and P\textsubscript{β}-phases.

Regarding DPT (4GSM) it has again not been possible to obtain a stable minimum for neither relative nor absolute widths of the layer thickness distribution.

3.1.5 Caillé Theory

The analysis of small-angle neutron scattering data is in the current section based on two versions of the Caillé theory, special Caillé theory and modified Caillé theory, which are used together with three different models of the scattering length density profile; 3GSM, 4GSM and GM. It should be noticed that GM will only be used together with MCT.

Fit parameter set

In SCT (3GSM) the head group, acyl-chain and water layer have again been taking into account (cf. Eq. (2.36), with \(F(q)\) given by Eq. (2.37) and \(<|f(q)|^2>\) given by Eq. (2.38)). The same goes for MCT (3GSM) (cf. Eq. (2.33) together with Eq. (2.31), where \(F(q)\) is given by Eq. (2.37)).

Likewise the methyl-terminus layer has additional been taking into account for SCT (4GSM) and MCT (4GSM), where \(F(q)\) and \(<|f(q)|^2>\) have been replaced by Eqs. (2.42) and (2.43). For MCT (GM) the head group and methyl-terminus layer are taken into account (cf. Eq. (2.33) together with Eq. (2.31), with \(F(q)\) given by Eq. (2.45)).

Fits performed with SCT (3GSM) require 8 different structural parameters, \(\tilde{\rho}, N, d_H, d_L, d_{\text{repeat}}, \sigma_H, \sigma_L\) and \(\eta_1\), together with a scala factor, \(\Gamma\), and a background value, \(I_{\text{bac}}\), for each SANS spectra. For MCT (3GSM) the number of structural parameters extends to 9 by \(N_{\text{diffuse}}\), which is the scala factor for the diffuse term.

Again, the number of fitted structural parameters increase when using 4GSM, for SCT the number is 11, consisting of \(\tilde{\rho}_L, \tilde{\rho}_M, N, d_H, d_L, d_M, d_{\text{repeat}}, \sigma_H, \sigma_L, \sigma_M\) and \(\eta_1\), while 12 for MCT. Note that the SCT (4GSM) parameter set has also been used for MCT (4GSM), though with the extension of \(N_{\text{diffuse}}\).

The parameter set for MCT (GM) consists of 7 fitted structural parameters, \(\rho_H, \rho_M, d_H, \sigma_H, \sigma_M, N, N_{\text{diffuse}}\) and \(\eta_1\) together with a background value for each spectra and a scale factor between each spectra.
The number of free structural parameters can again be reduced for 3GSM and 4GSM by letting the widths of the layer thickness distribution be relative to the layer thickness. Thus, $\sigma_\nu = \sigma_{\text{relative}} \cdot d_\nu$ and $\sigma_{\text{relative}}$ is the only fitted, where $\nu$ denotes the individual layers and $d_\nu$ the layers individual thickness. By doing this the number of free structural parameters in the scattering function for SCT (3GSM) and MCT (3GSM) can be reduced to 7 and 8 respectively. Thus, $\sigma_H$ and $\sigma_L$ are reduced to the single parameter $\sigma_{\text{relative}}$ for both models. The same procedure is possible with SCT (4GSM) and MCT (4GSM), though here the number of free structural parameters decreases to 9 and 10 respectively, with $\sigma_H$, $\sigma_L$, and $\sigma_M$ being reduced to $\sigma_{\text{relative}}$. Finally, it should be noticed that it is not possible to reduce the number of free structural parameters of MCT (GM).

**Fitting procedure**

The fitting of SCT and MCT follows the same procedure as describe for paracrystalline theory (cf. Section 3.1.3).

**Observations**

Regarding SCT$_r$ (3GSM), the scattering function is able to fit the third order diffraction peak for both DMPC-d$_{54}$ and DPPC-d$_{62}$ in the $L_\alpha$-phase, as it is the case with DPT$_r$ (3GSM) (Figure 3.6a,b). There is however an improvement, since SCT$_r$ (3GSM) also fits the zeroth order diffraction peak well. It should also be noticed that the fits obtained with SCT (3GSM) in the $P_{\beta^\prime}$-phase are significantly better, when compared to PT (3GSM) and DPT$_r$ (3GSM) (Figure 3.6e,f). Applying the diffuse term, $N_{\text{diffuse}}$, to SCT$_r$ (3GSM) only improve the fits to spectra of DPPC-d$_{62}$ in the $L_\alpha$-phase, whereas the quality of the fits to spectra of neither DMPC-d$_{54}$ nor DPPC-d$_{62}$ in the $P_{\beta^\prime}$-phase are improved (Figure 3.6g,h).

When the widths of the layer thickness distribution are let independent (absolute) no significant changes are observed in the fits with SCT$_a$ (3GSM) to spectra of both DMPC-d$_{54}$ and DPPC-d$_{62}$ in the $L_\alpha$-phase (Figure 3.7a,b). For spectra in the $P_{\beta^\prime}$-phase the quality of the fits with SCT$_a$ (3GSM) are worse than the ones performed with relative widths. This is equivalent for both DMPC-d$_{54}$ and DPPC-d$_{62}$, thus it is significantly pronounced for DPPC-d$_{62}$. Again, applying the diffuse term to SCT$_a$ (3GSM) does not improve the quality of fits to spectra of DPPC-d$_{62}$ in the $L_\alpha$-phase. For spectra of DMPC-d$_{54}$ in both phases the obtained fits are significantly worse for SCT$_a$ (3GSM), both with and without the diffuse term.

For MCT$_r$ (3GSM), the scattering function does not obtain the same agreement as SCT$_r$ (3GSM) (Figure 3.8). In general, MCT$_r$ (3GSM) underestimates the diffuse scattering intensity between the second and third order diffraction peak, and is not able to fit the peak shapes for all peaks. This is equivalent for spectra of DMPC-d$_{54}$ and DPPC-d$_{62}$ in both the $L_\alpha$-phase as well as the $P_{\beta^\prime}$-phase. MCT$_a$ (3GSM) has no significant effect on the quality of fits for spectra of both DMPC-d$_{54}$ and DPPC-d$_{62}$ in the $L_\alpha$-phase, and in the $P_{\beta^\prime}$-phase the fits have a lower quality than the ones performed with MCT$_r$ (3GSM).
Figure 3.6: The figure shows fits performed by using special Caillé theory with the 3GSM model. The fits are performed with relative widths of the layer thickness distribution function. The plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $L_\alpha$ with diffuse term (wd), (d) DPPC $L_\alpha$ wd, (e) DMPC $P_\beta$, (f) DPPC $P_\beta$, (g) DMPC $P_\beta$ wd, (h) DPPC $P_\beta$ wd.
Figure 3.7: The figure shows fits performed by using special Caillé theory with the 3GSM model. The fits are performed with absolute widths of the layer thickness distribution function. Again, the plots show (a) DMPC $L_\alpha$; (b) DPPC $L_\alpha$; (c) DMPC $L_\alpha$ with diffuse term (wd), (d) DPPC $L_\alpha$ wd, (e) DMPC $P_{\beta'}$, (f) DPPC $P_{\beta'}$, (g) DMPC $P_{\beta'}$ wd and (h) DPPC $P_{\beta'}$ wd.
3.1. ANALYSIS OF SMALL-ANGLE NEUTRON SCATTERING DATA (SANS)

Figure 3.8: The figure shows fits based on Modify Caillé theory with the 3GSM model. The fits are performed with relative widths of the layer thickness distribution function. The plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $P_{\beta'}$ and (d) DPPC $P_{\beta'}$

Figure 3.9: The figure shows fits based on Modify Caillé theory with the 3GSM model. The fits are performed with absolute widths of the layer thickness distribution function. The plots show (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $P_{\beta'}$ and (d) DPPC $P_{\beta'}$
Regarding SCT (4GSM) and MCT (4GSM) it has again not been possible to obtain stable minimums for neither relative nor absolute widths of the layer thickness distribution, which is due to a parameter correlation between the methyl-terminus layer thickness, \(d_M\), and the thickness of the acyl-chain layer, \(d_L\). It has neither been possible to obtain stable minimums for MCT (GM).

Finally, both versions of the Caillé theory together with the 3GSM model show the same high artificial values for \(\sigma_L\) in the \(P_{\beta'}\)-phase, which is caused by ripples. Regarding the thermal fluctuation between the layers, represented by the \(\eta_1\) parameter (\(\eta_1 \propto \sigma_\text{repeat}^2\) Figure 3.11), the values for the \(P_{\beta'}\)-phase is smaller than the ones for the \(L_\alpha\)-phase. This is expected when going from lipid-acyl-chain order to disorder.

3.1.6 Discussion of SANS Data and Comparison of Models

Because of the problems with obtaining stable fits, when using 4GSM and GM, only the 3GSM scattering length density profile will be taking into consideration in the discussion of SANS.
3.1. ANALYSIS OF SMALL-ANGLE NEUTRON SCATTERING DATA (SANS)

As previously discussed, it is not possible to obtain fits of adequately quality with PT \(_a\) (3GSM), which is why we also perform fits with absolute widths. A large decrease in \(\chi^2_{\text{reduced}}\) is observed for fits performed with PT \(_a\) (3GSM), while no general decrease in \(\chi^2_{\text{reduced}}\) is observed for fits performed with DPT \(_a\) (3GSM), SCT \(_a\) (3GSM) and MCT \(_a\) (3GSM).

When comparing the experimental measurements with the actual fits performed with paracrystalline, decoupled paracrystalline, special and modified Caillé theory (Figure 3.2-3.9), it is obvious that PT \(_a\) (3GSM) fits the details near the peaks better than both DPT (3GSM) and the two versions of Caillé theory, while SCT (3GSM) performs better fits in the region between the peaks, which is most pronounced for the \(P_{\beta'}\)-phase. One way to interpret this is that on the short length-scale the harmonic description underlying Caillé theory (Eq. (2.22)) is more adequate than the simple stochastic description underlying paracrystalline and decoupled paracrystalline theory.

Regarding the structural parameters for the \(L_\alpha\)-phase, the thickness variables, \(d\), \(d_B\), \(d_C\) and \(d_W\), do not deviate for the different different theories and models and are all within statistical error (Figure 3.10). \(d\) fluctuates around 61 Å for DMPC-\(d_{54}\) and 65 Å for DPPC-\(d_{62}\), while \(d_B\), \(d_C\) and \(d_W\) deviate more than \(d\). This is expected, since \(d\) can be determined directly from the scattering data independently of the chosen model. \(d_B\) fluctuates around 39 Å and 42 Å, \(d_C\) around 27 Å and 30 Å and \(d_W\) around 21 Å and 23 Å for DMPC-\(d_{54}\) and DPPC-\(d_{62}\) respectively. In general PT \(_a\) (3GSM) and MCT \(_a\) (3GSM) exhibit the largest deviations. In the \(P_{\beta'}\)-phase the differences become more pronounced, which is due to a larger scattering of \(d_B\) and \(d_C\). The parameters fluctuate around 62 Å for \(d\), 48 Å for \(d_B\), 35 Å for \(d_C\) and 15 Å \(d_W\) for DMPC-\(d_{54}\), while around 66 Å for \(d\), 56 Å for \(d_B\), 36 Å for \(d_C\) and 10 Å \(d_W\) for DPPC-\(d_{62}\).

The dynamical properties of the multi-lamellar lipid bilayer system can be studied through the fluctuation parameters shown in Figure 3.11. For the \(L_\alpha\)-phase \(\sigma_{\text{relative}}\) is determined to \(\sim 0.18\) and is about equal in size for all theories and models when fitted to scattering data obtained on DMPC-\(d_{54}\) and DPPC-\(d_{62}\), both with and without the diffuse term being applied. Only exceptions are paracrystalline theory with and without the diffuse term and modified Caillé theory, where \(\sigma_{\text{relative}}\) is respectively \(\sim 50\%\) larger and lower than \(\sim 18\). The \(P_{\beta'}\)-phase of DPPC-\(d_{62}\) follows the same pattern. \(\sigma_{\text{relative}}\) for the \(P_{\beta'}\)-phase of DMPC-\(d_{54}\) is determined to \(\sim 0.22\), both with and without the diffuse term applied, and again paracrystalline theory without the diffuse term and modified Caillé theory show significant deviations from the other theories and models.

From absolute widths of the layer thickness distribution \(\sigma_L\) can be determined to \(\sim 3\) for the \(L_\alpha\)-phase and \(\sim 4\) for the \(P_{\beta'}\)-phase (Figure 3.11), and is almost equal in size for all theories and models. Though, paracrystalline theory in the \(L_\alpha\)-phase shows a tendency of obtaining slightly lower values than decoupled paracrystalline and special Caillé theory, while no differences are observed for the \(P_{\beta'}\)-phase. Again, modified Caillé theory obtains values for \(\sigma_L\) that are significantly lower than \(\sim 3\) for both phases. For paracrystalline theory, a comparison of \(\sigma_W/d_W\) with \(\sigma_L/d_L\) shows that the water layer fluctuates more that the lipid bilayer relative to its thickness, with the only exception of DPPC-\(d_{62}\) in the \(P_{\beta'}\)-phase including the diffuse term. Intuitively the fluctuation parameters are expected to be equal for all theories and models, the larger \(\sigma_{\text{relative}}\) for paracrystalline theory implies therefore \(\sigma_{\text{relative}}\) is also covering \(\sigma_W\) for paracrystalline
theory.

(a) SANS scattering data obtained in the \( P_\beta' \)-phase

(b) SANS scattering data obtained in the \( L_\alpha \)-phase

Figure 3.11: Panel I show \( \chi^2_{\text{relative}} \) for Type I fits, while panel II to IV illustrate the fluctuation parameters obtained from SANS data on DMPC-\( d_{54} \) (closed symbols) and DPPC-\( d_{62} \) (open symbols). In panel I, II and IV the circles and squares correspond to the fits performed with relative and absolute widths of the layer thickness distribution respectively, while for panel III the squares and triangles correspond to the widths of the layer thickness distribution for the acyl-chain layer, \( \sigma_L \), and water layer, \( \sigma_W \), respectively. \( \sigma_{\text{Repeat}} \) in panel IV corresponds to the width of the Gaussian distribution for the repeat distance.

The total size of fluctuations can also be compared by the fluctuation of the repeat unit, \( \sigma_{\text{repeat}} \). For PT, \( \sigma_{\text{repeat}} \) is given by \( (\sum_\nu \sigma^2_\nu)^{1/2} \), with \( \nu = \text{H}, \text{L, W} \), while for SCT and MCT \( \sigma_{\text{repeat}} \) is given by \( d\sqrt{\eta_1[\gamma + \log(\pi)]/\pi \sqrt{2}} \) (cf. Eq. (2.30)). In general \( \sigma_{\text{repeat}} \) is almost equal in size for DPT and SCT, while significantly larger for PT. This is reasonable, since a decoupling of the form and structure factors correspond to neglecting parts of the system fluctuations. For MCT (3GSM), \( \sigma_{\text{repeat}} \) is similar to DPT and SCT. Unfortunately the fluctuation parameters can not discriminate between static distortions and dynamical fluctuations of the multi-lamellar lipid bilayer system (Figure 3.11).

Based on the above it can be concluded that the best fits to small-angle neutron scattering data are performed with PT\(_a\) (3GSM) and SCT (3GSM). The physical assumption that widths of the thickness distribution are relative to the thickness of each
layer can furthermore be concluded non-valid for non-decoupled paracrystalline theory, which might be due to a strong coupling between all functions in the system. The physical assumption seems in comparison valid for decoupled theories, which is due to their ability of obtaining reliable fits with relative widths of the thickness distribution. The validity might be explained by the lack of coupling between water layer fluctuations and fluctuations within the bilayer.

In the $L_\alpha$-phase SCT (3GSM) performs smaller $\chi^2_{\text{reduced}}$ values than PT$_r$ (3GSM), but when changing to absolute widths of the thickness distribution PT$_a$ (3GSM) obtains slightly better fits than SCT (3GSM), both with and without the diffuse term applied. By applying the diffuse term, PT (3GSM) performs fits with slightly lower $\chi^2_{\text{reduced}}$ values, while it is impossible to determine the impact of $N_{\text{diffuse}}$ on SCT (3GSM).

In the $P_\beta'$-phase, $\chi^2_{\text{reduced}}$ for the high-$q$ spectra furthermore shows that PT$_a$ performs slightly better fits than SCT$_a$ and SCT$_r$ for DMPC-d$_{54}$. Both theories take however only the structural details perpendicular to the bilayer plane into account, while leaving out the ones parallel to the bilayer plane. This is why neither PT nor SCT perform acceptable fits in the $P_\beta'$-phase, they do simply not account for the ripples. Applying the diffuse term reveal no general tendencies in the $P_\beta'$-phase. For PT, the application of the diffuse term causes a significant reduction in $\chi^2_{\text{reduced}}$ for high-$q$ spectra, while it causes a larger increase in $\chi^2_{\text{reduced}}$ for SCT. Applying the diffuse term to DPT, reveal both large increases and decreases in $\chi^2_{\text{reduced}}$.

Finally, it should be said, that even thought PT and SCT in general perform the best fits to SANS spectra, the theory best describing the bilayer system can not be determined from the quality of the fits due to lack of resolution. Accordingly the most appropriate physical assumption for describing multi-lamellar bilayer systems can not be determined. It is neither possible to determine whether applying a diffuse term to SCT have an impact or not on the fits, while it should always be applied to PT. Based on this it can be concluded, that SCT is able to account for the diffuse scattering, while PT can not describe the bilayer system without including the diffuse scattering from uncorrelated bilayers. Regarding which model for the scattering length density profile best describing the deuterated bilayer, it is obviously that 3GSM is the best and only model, since 4GSM and GM can not obtain stable fits, which might be caused by the weak contrast between the acyl-chains and the methyl-terminus.

3.2 Analysis of Small-Angle X-ray Scattering Data (SAXS)

The SAXS analysis is based on small-angle x-ray scattering data obtained on multi-lamellar samples of DMPC and DPPC by Jan S. Petersen (University of Århus), Rogert Bauer (The Royal Veterinary and Agricultural University), Tommy Nylander and Jan Kharkar (Lund University) on a SAXS camera connected to a rotating copper anode x-ray generator placed at Risø National Laboratory. The lipids are dissolved in D$_2$O to a concentration of 20 wt% lipid and obtained at a temperature $T = 20.6^\circ$C and 40.6$^\circ$C for DMPC and $T \sim 25^\circ$C and 55$^\circ$C for DPPC. The temperatures correspond to the $P_\beta'$ and $L_\alpha$-phase for DMPC and the $L_\beta'$ and $L_\alpha$-phases for DPPC. The SAXS data
are obtained at a wavelength of 1.542 Å and a sample-to-detector distance of \( \sim 1 \) m.

The SAXS data are analyzed in the same way as the SANS data.

### 3.2.1 Instrumental Resolution and Deconvolution of SAXS Scattering Data

A nonlinear least-square fitting routine has also been used in the analysis of small-angle x-ray scattering data, and the smearing has again been taken into account as suggested by Pedersen et al. (Pedersen et al., 1990).

For SAXS scattering data \( \Delta \lambda/\lambda \) is negligible, whereby it is only the instrumental geometric set-up that contributes to the smearing of the experimental data. Data points with \( q < 0.07 \) Å have not been taking into account, since there are problems with the \( \lambda/2 \) peak and uncertainties caused by large background subtraction. This regards both DMPC and DPPC samples. For the spectrum obtained on DMPC in the \( P'_{\beta} \)-phase, it has however been necessary to cut the data points above \( q \sim 0.33 \) Å\(^{-1}\), due to problems with the background and fitting procedure.

### 3.2.2 Accuracy of SAXS Scattering Data

Two order of diffraction peaks, \((1,0)\) and \((2,0)\), have been observed for SAXS spectra obtained on DPPC in the \( L_\alpha \)-phase (Figure 3.12b,d), while four order of diffraction peaks have been observed in the \( L_\beta \)-phase. The \((1,0)\), \((2,0)\), \((3,0)\) and \((4,0)\) peaks are illustrated in Figure 3.12f,h. For SAXS spectra obtained on DMPC in both the \( L_\alpha \) and \( P'_{\beta} \)-phases two order of diffraction peaks are observed. The \((1,0)\) and \((2,0)\) peaks are illustrated in Figure 3.12a,c,e,g. From a brief visual inspection of Figure 3.12 it can be seen that the diffraction peaks follow a power-law behavior, which might be caused by a less pronounced smearing due to a negligible wavelength spread. The \((n,1)\) ripple peaks in the \( P'_{\beta} \)-phase can also very easily be discerned in the SAXS spectrum performed on DMPC, where they appear as shoulders on the \((1,0)\) and \((2,0)\) order diffraction peaks (Figure 3.12). The \((2,1)\) ripple peak is comparable in intensity with the \((2,0)\) order diffraction peak, and they occur as one very broad single peak. As with SANS spectra, no higher order ripple peaks \(((0,n) \ n > 1)\) are detected in the SAXS spectrum for DMPC. Finally, the SAXS scattering data also shows an increasing width of the diffraction peaks as the diffraction order increases, as with SANS and again, this reflects the quasi-long range order of the multi-lamellar lipid bilayer system characterizing liquid crystals.

### 3.2.3 Paracrystalline Theory

The analysis of small-angle x-ray scattering data based on paracrystalline theory is in the present ph.d. thesis used together with the 3GSM and 4GSM scattering length density profile models.
3.2. ANALYSIS OF SMALL-ANGLE X-RAY SCATTERING DATA (SAXS)

Fit parameter set

The paracrystalline parameter set mentioned in the SANS section is also used on SAXS spectra (cf. Section 3.1.3). This counts for both relative and absolute widths of the layer thickness distribution. It should however be noticed that only one interval of \( q \)-values has been used in the fits to SAXS data.

Fitting procedure

No special fitting procedure has been performed on SAXS data due to the use of only one interval of \( q \)-values.

Observations

Both PT\(_r\) (3GSM) and PT\(_r\) (4GSM) fit the first and second order diffraction peaks well, while they do not fit the diffuse scattering between the first and second order diffraction peaks (Figure 3.12).

For DMPC in the \( L_\alpha \) and \( P_\beta \)-phases and DPPC in the \( L_\alpha \) and \( L_\beta' \)-phases it can furthermore be seen that both models overestimate the scattered intensity in the tail region of the first order diffraction peak. It should also be noticed that the scattered intensity for the fourth order diffraction peak is overestimated by PT\(_r\) (3GSM) in the DPPC spectra, cause even though the peak does not appear in the spectra, it is still present in the fitted function of PT\(_r\) (3GSM). PT\(_r\) (4GSM) has in contrast a tendency of underestimating the third order diffraction peak in the SAXS data.

To account for the scattered intensity between the first and second order diffraction peaks, a diffuse term, \( N_{\text{diffuse}} \), has again been added. This term adequately accounts for the diffuse scattering between the first and second order diffraction peaks (Figure 3.12e,g). When comparing PT\(_r\) (3GSM) and PT\(_r\) (4GSM) it can furthermore be seen that PT\(_r\) (4GSM) in general produces better fits to the SAXS spectra than PT\(_r\) (3GSM), both with and without the diffuse term being applied.

The mismatch between the fitted scattering function and the DMPC spectrum in the \( P_\beta \)-phase might be caused by the (1,1) and (2,1) ripple peaks as explained by Matuoka et al. (Matuoka et al., 1990). In the DMPC spectra the (2,1) peak is comparable in intensity with the (2,0) order diffraction peak and are shown as one broad single peak. The peaks can not be separated because of very small differences in \( q \)-values (Figure 3.12e,g). This broadening gives an artificial increase in the fluctuation of the system, which is why the real fluctuation is consequently overestimated (Fig 3.16). The overestimation influences the value of \( \sigma_{\text{relative}} \), which is larger for the \( P_\beta \)-phase than the \( L_\alpha \)-phase. For DPPC spectra the value of \( \sigma_{\text{relative}} \) increases, when the system changes from the \( L_\beta' \)-phase to the \( L_\alpha \)-phase. It should also be noticed that the ripple peaks do not influence the fitted position of the peaks, which counts for both PT\(_r\) (3GSM) and PT\(_r\) (4GSM).

Regarding PT\(_a\) (3GSM) and PT\(_a\) (4GSM) it has not been possible to obtain stable minima. Therefore the results from fits to SANS spectra are used for each layer in the determination of the scala factor to \( \sigma_{\text{relative}} \). Thus, in the first set \( \sigma_L = \sigma_{\text{relative}} \cdot d_L \) then \( \sigma_W = 1.5 \cdot \sigma_{\text{relative}} \cdot d_W \) and finally \( \sigma_M = \sigma_{\text{relative}} \cdot d_M \), where \( \sigma_H \) is set equal to
Figure 3.12: The figure shows fits performed when using paracrystalline theory with relative widths of the layer thickness distribution function. The dotted line is (4GSM) and the dash line is (3GSM). The plots show (a) DMPC \( L_\alpha \), (b) DPPC \( L_\alpha \), (c) DMPC \( L_\alpha \) with diffuse term (wd), (d) DPPC \( L_\alpha \) wd, (e) DMPC \( P_\beta' \), (f) DPPC \( L_\beta' \), (g) DMPC \( P_\beta' \) wd and (h) DPPC \( L_\beta' \) wd.
zero. Fits performed with the corrected PT\(_r\) (3GSM) show a decrease in \(\chi^2_{\text{reduced}}\) close to 20 % for spectra of DMPC in the \(P_{\beta}\)-phase and DPPC in \(L_{\beta}\)-phase. The decrease in \(\chi^2_{\text{reduced}}\) is however not observed for spectra of neither DMPC nor DPPC in the \(L_{\alpha}\)-phase. The corrected parameter set will therefore not be used any further in the analysis of SAXS data.

As implied by 3GSM and 4GSM, the head and water regions are clearly separable in the simple geometric modelling of the form factor. This is obviously a simplification, since the hydration of the head group tends to smooth out the boundary between the two regions, but it gives rise to a rather strong coupling between \(d_H\) and \(d_W\), which makes it difficult to do reliable estimates. In practice, this means the values of \(d_H\) and \(d_W\) are quite sensitive to small changes in the fitting procedure, whereas the value of the total hydrophilic layer thickness, \(d_A = 2d_H + d_W\), is stable and displays less scattering. This coupling gives specially implications for SAXS, which is why the head group width, \(d_H\), is derived from SANS data and set to a value of 7.2 Å for all fits of SAXS data.

![Figure 3.13](image)

Figure 3.13: The figures illustrates the repeat distance, \(d\), the bilayer thickness, \(d_B\), the hydrophobic core thickness, \(d_C\), and the water layer thickness, \(d_W\), obtained from SAXS spectra on DMPC (closed symbols) and DPPC (open symbols).

Finally, it can be seen from the structural parameters that PT\(_r\) (4GSM) gives a larger hydrophobic part of the bilayer than PT\(_r\) (3GSM). This can be explained by splitting the hydrophobic part into two parts with different scattering length densities,
\[ \rho. \] The separation enhances the acyl-chain's scattering length density, \( \tilde{\rho}_L \), to be closer to the head group's scattering length density, \( \tilde{\rho}_H \), whereby a larger hydrophobic part occurs.

### 3.2.4 Decoupled Paracrystalline Theory

The analysis of small-angle x-ray scattering data based on decoupled Paracrystalline theory are used with the 3GSM and 4GSM scattering length density profile models.

**Fit parameter set**

Again, the parameter set for decoupled paracrystalline theory performed on SANS spectra have been used in the analysis of SAXS spectra (cf. Section 3.1.4), and as for paracrystalline theory only one interval of \( q \)-values has been used.

**Fitting procedure**

Again, no special fitting procedure has been performed for the SAXS data, which is due to the use of only one interval of \( q \)-values.

In 4GSM the boundary between the acyl-chain layer and the methylene-terminus is impossible to separate. However, in contrast to PT_\( r \) (4GSM), the coupling between \( d_L \) and \( d_M \) does not influence the total width of the hydrophobic part \( 2(d_L + d_M) \), but affects only the ratio between them. In the DPT fitting a strong correlation between several parameters leads to an unphysical large width of the hydrophobic part of the bilayer. To overcome this, a parameter change from \( d_L, d_M, \tilde{\rho}_L \) and \( \tilde{\rho}_M \) to \( (d_L + d_M), d_M, \tilde{\rho}_L = (\tilde{\rho}_L - 1) \) and \( \tilde{\rho}_M = (\tilde{\rho}_M - \tilde{\rho}_L) \) is made, making the strong coupling between \( d_L \) and \( d_M \) disappear. Based on this it is now possible to fit the total width of the hydrophobic part and the methyl-terminus, while the strong correlation between \( d_M \) and the scattering length density still persists. This give nevertheless a well defined \( \chi^2 \) reduced minimum.

**Observations**

Regarding DPT_\( r \) (3GSM) and DPT_\( r \) (4GSM), the scattering function perform fits of good quality for both DMPC and DPPC in the \( L_\alpha \)-phase. A visual inspection of Figure 3.14 also shows that the scattering function both overestimates the amplitude of the first order diffraction peak \( (1,0) \) as well as underestimates the peak’s width (Figure 3.14a,b,e,f). The opposite is the case for the second order diffraction peak \( (2,0) \). For DMPC in the \( P_\beta' \)-phase and DPPC in the \( L_\beta' \)-phase it can furthermore be seen that DPT_\( r \) (4GSM) obtains better agreement for the diffused scattered intensity between the first and second order diffraction peaks than DPT_\( r \) (3GSM). For DPPC in the \( L_\beta' \)-phase neither DPT_\( r \) (3GSM) nor DPT_\( r \) (4GSM) yield intensity in the fourth order diffraction peak.

Applying the diffuse term, \( N_{\text{diffuse}} \), to DPT (3GSM) and DPT_\( r \) (4GSM) do not cause significant changes for any of the models (Figure 3.14 and 3.16).
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Figure 3.14: The figure shows fits performed when using decoupled paracrystalline theory with relative widths of the layer thickness distribution function. The dotted line is 4GSM and the dashed line is 3GSM. The plots shows (a) DMPC $L_{\alpha}$, (b) DPPC $L_{\alpha}$, (c) DMPC $L_{\alpha}$ with diffuse term (wd), (d) DPPC $L_{\alpha}$ wd, (e) DMPC $P_{\beta}$, (f) DPPC $L_{\beta'}$, (g) DMPC $P_{\beta'}$ wd and (h) DPPC $L_{\beta'}$ wd.
Again, a mismatch can be observed between the scattering function and the SAXS spectra for DMPC in the $P_{\beta'}$-phase, while again is most likely caused by the (1,1) and (2,1) ripple peaks.

From the structural parameters in Figure 3.13, it can also be seen that the width of the hydrophobic part, $d_C$, has the same development as when using PT and that fitting DPT$_r$ (4GSM) to all SAXS spectra gives a slightly larger width than fits performed with DPT$_r$ (3GSM). Only exception is for DPPC in the $L_\alpha$-phase, where the opposite happens.

### 3.2.5 Caillé Theory

The analysis of small-angle x-ray scattering data is based on two versions of the Caillé theory; special Caillé theory Eq. (2.36) and modified Caillé theory Eq. (2.50), and they are used together with the 3GSM, 4GSM and GM models. Again, please notice GM is only used together with MCT.

#### Fit parameter set

For special and modified Caillé theory the parameter set from SANS (cf. Section 3.1.5) has again been used. And as for paracrystalline and decoupled paracrystalline theory only one interval of $q$-values has been used.

#### Fitting procedure

No special fitting procedure has been used for Caillé theory performed on SAXS data, which again is due to the use of only one interval of $q$-values.

In the fitting procedure of 3GSM and 4GSM in SCT and MCT, the $d_H$ has been kept constant at 7.2 Å to prevent cross correlations between the water and head group layers, as mention in Section 3.2.3 and 3.2.4. The parameter change used on DPT (Section 3.2.4) has also been used on 4GSM to prevent strong coupling between the acyl-chain and the methyl-terminus. The parameter change from $d_L$, $d_M$, $\tilde{\rho}_L$ and $\tilde{\rho}_M$ to ($d_L + d_M$), $d_M$, $\tilde{\rho}_L = (\tilde{\rho}_L + 1)$ and $\tilde{\rho}_M = (\tilde{\rho}_M - \tilde{\rho}_L)$ makes it possible to fit both the total width of the hydrophobic part and the width of the methyl-terminus. The strong correlation between $d_M$ and the scattering length density is not affected by the parameter change, whereas it is still present.

#### Observations

Regarding SCT$_r$ (3GSM) and SCT$_r$ (4GSM), the scattering function performs fits very similar to the ones obtained by decoupled paracrystalline theory for DMPC spectra in both the $L_\alpha$ and $P_{\beta'}$-phases. Thus, it should be noticed that the peaks are better reproduced with SCT (Figure 3.15a,e).

SCT$_r$ (3GSM) and SCT$_r$ (4GSM) give fits of equally good quality. For SAXS spectra of DPPC in both the $L_\alpha$ and $L_{\beta'}$-phases, (Figure 3.15), SCT$_r$ (3GSM) and SCT$_r$ (4GSM) obtain better fits than DPT. For DPPC in the $L_{\beta'}$-phase SCT$_r$ (3GSM) and SCT$_r$ (4GSM) also show a tendency of underestimating the scattering intensity
3.2. ANALYSIS OF SMALL-ANGLE X-RAY SCATTERING DATA (SAXS)

Figure 3.15: The figure shows fits performed when using special Caillé theory with relative widths of the layer thickness distribution. The dotted line is 4GSM and the dashed line is 3GSM. The plots shows (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $L_\alpha$ with diffuse term (wd), (d) DPPC $L_\alpha$ wd, (e) DMPC $P_{\beta'}$, (f) DPPC $L_{\beta'}$, (g) DMPC $P_{\beta'}$ wd and (h) DPPC $L_{\beta'}$ wd.
between the first and second order diffraction peaks while overestimating the scattering intensity in the tail region of the peaks (Figure 3.15f). SCT$_r$ (3GSM) estimates however the amplitude of the third order diffraction peak better that SCT$_r$ (4GSM), while overestimates the width of the peak. For the fourth order diffraction peak none of the models give satisfactory fits, thus SCT$_r$ (3GSM) overestimates the peak while SCT$_r$ (4GSM) underestimates it. In relation to the diffuse scattering SCT$_r$ (4GSM) also obtains better fits between the peaks.

Applying the diffuse term, $N_{\text{diffuse}}$, to SCT$_r$ (3GSM) and SCT$_r$ (4GSM) has only a small effect on the fitting results (Figure 3.15c,g,d,h).

For MCT$_r$ (3GSM) and MCT$_r$ (4GSM), the scattering function performs fits very similar to SCT$_r$ (3GSM) and SCT$_r$ (4GSM) (Figure 3.17). The MCT fits are however slightly better than SCT for DMPC in the $P_{\beta'}$-phase and DPPC in the $L_{\beta'}$-phase. Specially the fourth order diffraction peak in the $L_{\beta'}$-phase of DPPC is better obtained by MCT. For the $L_{\alpha}$-phase of both DMPC and DPPC, MCT$_r$ obtains slightly better fits than SCT$_r$ and MCT$_r$ does generally not fit the diffuse scattering as well as SCT$_r$.

Fits performed with MCT (GM) on DMPC and DPPC are hardly distinguishable from fits performed with MCT$_r$ (3GSM) and MCT$_r$ (4GSM). Only a slightly variation in $q$-values above 0.25 Å$^{-1}$ for DPPC in the $L_{\alpha}$-phase can be observed. MCT (GM) fits furthermore the $(4,0)$ peak better than both MCT$_r$ (3GSM) and MCT$_r$ (4GSM).

Regarding SCT (3GSM), SCT (4GSM), MCT (3GSM) and MCT (4GSM) it has again not been possible to obtain stable minimums for absolute widths of the layer thickness distribution.

The structural parameters, obtained from fits based on Caillé theory, also show that the width of the hydrophobic part, $d_C$, has the same development as when using paracrystalline and decoupled paracrystalline theory (Figure 3.13).

### 3.2.6 Discussion of SAXS Data and Comparison of Models

In the discussion of SAXS only fits performed with relative widths of the layer thickness distribution will be taken into account, since it has not been possible to obtain stable fits with absolute widths.

The best results of $\chi^{2}_{\text{reduced}}$ are obtained by fitting SAXS scattering data in the $L_{\alpha}$-phase with Caillé theory. Applying a diffuse term to the different theories and models do not cause any significant changes, when the fluctuations are correlated to $\sigma_{\text{relative}}$.

When comparing the actual fits to the experimental data in terms of PT, DPT, SCT and MCT it is obvious that PT does not fit the diffuse scattering between the peaks, while both SCT and MCT do. SCT and MCT also generally fit the shape of the peaks better than DPT and PT, in mentioned order. In relation to the scattering length density profile, 3GSM and 4GSM obtain same quality of fits, though with the exception of DPPC in the $L_{\beta'}$-phase, where 4GSM performs better than 3GSM. This counts for all theories.

The structural parameters (Figure 3.13) show a repeat distance, $d$, that is nearly constant for models within the same phase. The obtained values are 66 and 62 Å for DMPC in the $P_{\beta'}$ and $L_{\alpha}$-phase respectively and 64 and 66 Å for DPPC in $L_{\beta'}$ and $L_{\alpha}$-phases respectively. Regarding the thickness of the hydrophobic part, $d_C = 31$ Å
3.2. ANALYSIS OF SMALL-ANGLE X-RAY SCATTERING DATA (SAXS) \[69\]

**Figure 3.17:** The figure shows fits performed when using modified Caillé theory with relative widths of the layer thickness distribution function. The dotted line is 4GSM and the dashed line is 3GSM. The plots shows (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $P_{\beta'}$ and (d) DPPC $L_{\beta'}$-phase.

**Figure 3.18:** The figure shows fits performed when using modified Caillé theory with the GM model. The plots shows (a) DMPC $L_\alpha$, (b) DPPC $L_\alpha$, (c) DMPC $P_{\beta'}$ and (d) DPPC $L_{\beta'}$-phase.
for DPPC in $L_\beta$ which is $\sim 4$ Å thicker when compared to DPPC in the $L_\alpha$-phase. This is expected due to the all trans conformation of the acyl-chain in the $L_\beta$-phase, which changes to fluid in the $L_\alpha$-phase, where e.g. gauche conformations are introduced to the acyl-chain. Same tendency are found between the $P_\beta$ and the $L_\alpha$-phase for DMPC. From the SAXS scattering data it can furthermore been seen that 4GSM performs larger values of $d_C$ than 3GSM, with only a few exceptions. One of the exceptions is DPT, SCT and MCT in the $L_\alpha$-phase of DPPC, where 3GSM results in an $\sim 3$ Å larger hydrophobic part. For fits performed on DMPC and DPPC in the $L_\alpha$-phase both SCT and MCT obtain smaller values of $d_C$ than PT and DPT, while MCT obtains a slightly larger hydrophobic thickness than SCT for DPPC in the $L_\beta$-phase. For DMPC in the $P_\beta$, DPT, SCT and MCT obtain $d_C$ values similar in size and significantly lower than the values obtained by PT. Due to the fixed head group thickness the bilayer thickness, $d_B$, is correlated to $d_C$.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3_16.png}
\caption{The figure shows $\chi^2_{relative}$ and fluctuation parameters obtained from SAXS scattering data of DMPC (closed symbols) and DPPC (open symbols) are presented in panel I and II/III respectively. $\sigma_{relative}$ corresponds to the relative width of the layer thickness distribution in panel II, while $\sigma_{Repeat}$ corresponds to the width of the Gaussian distribution function for the unitcell in panel III.}
\end{figure}

The fluctuation parameters in Figure 3.16 show a $\sigma_{relative}$ about equal in size for DMPC and DPPC in the $L_\alpha$-phase and a 4GSM model apparently performing larger $\sigma_{relative}$ values than 3GSM for DPPC. This is equivalent for all theories, though decoupled paracrystalline theory performs larger fluctuations for DMPC than PT, SCT and MCT, both with and without the diffuse term. PT performs the smallest fluctuations for DPPC. Intuitively $\sigma_{relative}$ is expected to be equal for all theories and models, but
as a consequence of PT’s poor ability to fit the SAXS scattering data it seems that PT might underestimates the fluctuations in order to fit the amplitude of the peaks.

Regarding the total size of fluctuations, quantified by $\sigma_{\text{repeat}}$, fits performed with Caillé theory are almost comparable in size and somewhat larger than fits performed with both paracrystalline and decoupled paracrystalline theory, which is in contrast to the results obtained on SANS data. $\sigma_{\text{repeat}}$ for DPT and PT are also similar in size, though with the exception of DPPC in the $L_{\beta'}$-phase, where the total size of fluctuations for DPT are in between PT and the ones obtained with Caillé theory. None of the theories and models perform acceptable fits for the DMPC spectra in the $P_{\beta'}$-phase, which is most likely due to the presence of ripple peaks.

From the quality of fits it can be concluded that special and modified Caillé theory generally perform very good fits to small-angle x-ray scattering data and that Caillé theory gives the best description of multi-lamellar bilayer systems when compared to paracrystalline theory. This means the underlaying harmonic description of the free energy in Caillé theory is better in describing the physical behavior of the bilayer fluctuations than the non-decoupled purely stochastic description of paracrystalline theory. SCT also performs slightly, but not significantly, better fits than MCT, whereas there is no basis for a general conclusion on which version of the Caillé theory that is best to use when performing fits to SAXS.

In relation to the structural parameters it should be remembered that Caillé theory does not take the in-plane shear module into account. Caillé theory is therefore not adequate for the $L_{\beta'}$-phase. The theory is however still able to obtain stable fits to the $L_{\beta'}$-phase, though generally with significant systematical deviations. The structural parameters should accordingly be taken with reservation. Regarding the diffuse term, it should always be added to paracrystalline theory, as with SANS, while it seems to have no effect on DPT and SCT in SAXS.

All models of the scattering length density profile describe the multi-lamellar bilayer systems equally good, but due to the lower number of free structural parameters, and the fact that 4GSM does not give significantly better fits than 3GSM it can be concluded that 3GSM is the best scattering length density profile model to use on x-ray scattering data. Only exception is MCT, where the GM model can be used just as well as 3GSM. Finally, it should be noticed that for SAXS spectra obtained on samples with a higher resolution and a better identified methyl-terminus in the electron density profile than the ones used in the present work 4GSM might be more suitable than 3GSM.

### 3.3 Area per Lipid Molecule

As illustrated in Figures 3.19 and 3.20, the calculated values of the average interfacial area per lipid molecule, $A$, are larger when based on Eq. (2.55) than Eq. (2.56).

For SANS data the difference in calculated values of $A$ might be caused by the fact that the volume of the head group, $V_H$, is defined from x-ray studies. The value of $V_H$ therefore might not be correct, since subgroups of the lipid bilayer are defined differently for neutron and x-ray due to differences in scattering length density.

For SAXS the difference in calculated values of $A$ can be caused by the lack of
CHAPTER 3. ANALYSIS OF DATA AND COMPARISON OF METHODS

Figure 3.19: The figure shows the cross-sectional area and number of water molecules per lipid molecule for DMPC-d$_{54}$ (closed symbols) and DPPC-d$_{62}$ (open symbols) obtained by SANS. Circles and squares correspond to values calculated from the bilayer thickness while triangles and diamonds correspond to values calculated from half of the hydrophobic core. Circles and triangles arise from fits using relative widths of the thickness distribution while squares and diamonds arise from fits using absolute widths of the thickness distribution.

ability to define the border between the head group and the hydrophobic core. It has therefore not been possible to fit the thickness of the head group, whereas the thickness of the hydrophobic core might be too small and $A_{dC/2}$ overestimated.

Variations in $A$ can also be observed for the different models. MCT$_r$ (3GSM) performed on SANS obtains significantly smaller $A_{d_B}$ values than PT, DPT and SCT. For SAXS the scattering length density profile 4GSM generally obtains smaller $A$ values than 3GSM. This is not unexpected since the bilayer thickness, $d_B$, is somewhat larger for 4GSM than 3GSM. Finally, it can be seen that both SCT and MCT obtain larger $A$ values than PT and DPT.

3.4 Comparison of Results

In this section only results obtained on the $L_\alpha$-phase are taking into account, due to the fact that Caillé is only valid for this phase and that no x-ray scattering data are obtained on DPPC.

For all models the thickness variables are within statistical error. This was specially expected for the repeat distance, $d$, since it can be determined directly from all spectra, independently of chosen model.

When comparing the thickness variables obtained by SANS and SAXS, some differences can be observed. For SANS spectra obtained on DMPC-d$_{54}$ and DPPC-d$_{62}$ in the $L_\alpha$-phase the repeat distance, $d$, is measured roughly to 61 Å and 65 Å. In comparison $d$ is measured to 62 and 66 Å when performed on SAXS scattering data. The small differences in Å might be due to differences in the experimental conditions for SANS and SAXS. E.g. is the multi-lamellar lipid dissolved in a D$_2$O buffer for SANS while it is pure D$_2$O for SAXS. The SANS and SAXS spectra are also obtained at different
3.4. COMPARISON OF RESULTS

Figure 3.20: The figure shows the cross-sectional area and number of water molecules per lipid molecule for DMPC (closed symbols) and DPPC (open symbols) obtained by SAXS. Circles correspond to values calculated from the bilayer thickness, while triangles correspond to values calculated from half of the hydrophobic core.

In relation to the thickness of the hydrophobic core, $d_C$, SANS spectra obtain values that are several Å larger than the SAXS values in the $L_\alpha$-phase. The difference in $d_C$ can be explained by the fact that the carbonyl-oxy group is included in the hydrophobic core for SANS due to a comparable size of the scattering length density for oxygen, carbon and deuterium, while the carbonyl-oxy group is included in the head group for SAXS because of a larger electron density for oxygen than carbon. By taking the length of the acyl-oxy group (∼2.5 Å per acyl-chain) into account, the differences between SANS and SAXS $d_C$ values become acceptable.

In general, the bilayer thickness, $d_B$, is larger for 4GSM than 3GSM, when performed on SAXS scattering data. However, SAXS $d_B$ values are again several Å smaller than the SANS values. This might be caused by the fact that it is not possible to fit the head group thickness, $d_H$, for SAXS, whereas the value has been set to 7.2 Å, which is a rough average taken from SANS. Obviously this is not an optimal value, since it does not take the carbonyl-oxy group into account, but it is our best guess. Again, by taking the length of the acyl-oxy group into account the differences between SANS and SAXS $d_B$ values become acceptable.

Despite differences in definition the thickness variables are also in reasonable agreement with values obtained by other groups, as illustrated in Table 3.1. For the hydrophobic core Nagle and Tristram-Nagle (2000) obtained a $d_C = 26.2$ Å and G. Pabst and Rappolt (2003) a $d_C = 26.4$ Å. Both studies were performed on DMPC at T = 30°C. By taking the temperature dependencies, anomalous swelling, and the length of the carbonyl-oxy group into account, the obtained $d_B$ values in present ph.d. thesis are in reasonable agreement with Nagle and Tristram-Nagle (2000) and G. Pabst and Rappolt (2003). From Table 3.1 it can also be seen that the reported $d_B$ values have large divergences, depending on the method used in the determination of the structural parameters.
### Table 3.1: The table shows the structural parameters from Janiak et al. (Janiak et al., 1976, 1979), Gordeliy et al. (1996), Nagle and Tristram-Nagle (2000) and G. Pabst and Rappolt (2003). It should be noticed that the references have different definitions of the thicknesses, also when compared to our definitions.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Temp (°C)</th>
<th>$V_L$ ($\text{Å}^3$)</th>
<th>$d$ (Å)</th>
<th>$A$ (Å$^2$)</th>
<th>$d_C$ (Å)</th>
<th>$d_B$ (Å)</th>
<th>$d_W$ (Å)</th>
<th>$n_W$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC (Janiak et al., 1976, 1979)</td>
<td>25</td>
<td>63.1</td>
<td>49.2</td>
<td>48.4</td>
<td>14.7</td>
<td>12.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC (Nagle and Tristram-Nagle, 2000)</td>
<td>20</td>
<td>1144</td>
<td>63.5</td>
<td>47.9</td>
<td>34.4</td>
<td>52.4</td>
<td>11.1</td>
<td>12.6</td>
</tr>
<tr>
<td>DMPC (Janiak et al., 1976, 1979)</td>
<td>20</td>
<td>64.8</td>
<td>47.4</td>
<td>44.5</td>
<td>20.3</td>
<td>15.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPC (Gordeliy et al., 1996)</td>
<td>20</td>
<td>68.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPC (Nagle and Tristram-Nagle, 2000)</td>
<td>30</td>
<td>1101</td>
<td>62.7</td>
<td>59.6</td>
<td>26.2</td>
<td>44.2</td>
<td>18.5</td>
<td>25.6</td>
</tr>
<tr>
<td>DMPC (G. Pabst and Rappolt, 2003)</td>
<td>30</td>
<td>1101</td>
<td>63.4</td>
<td>59.4</td>
<td>26.4</td>
<td>46.8</td>
<td>18.5</td>
<td>18.2</td>
</tr>
<tr>
<td>DMPC (Janiak et al., 1976, 1979)</td>
<td>40</td>
<td>60</td>
<td>62.2</td>
<td>35.5</td>
<td>24.5</td>
<td>25.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPC (Gordeliy et al., 1996)</td>
<td>39</td>
<td>63.2</td>
<td></td>
<td></td>
<td></td>
<td>35.1</td>
<td>28.1</td>
<td></td>
</tr>
<tr>
<td>DPPC (Nagle and Tristram-Nagle, 2000)</td>
<td>50</td>
<td>1232</td>
<td>67</td>
<td>64</td>
<td>28.5</td>
<td>46.5</td>
<td>20.5</td>
<td>30.1</td>
</tr>
<tr>
<td>DPPC (Janiak et al., 1976, 1979)</td>
<td>50</td>
<td>60</td>
<td>68.4</td>
<td>35.4</td>
<td>24.6</td>
<td>27.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC (Gordeliy et al., 1996)</td>
<td>55</td>
<td>67</td>
<td></td>
<td></td>
<td></td>
<td>41.5</td>
<td>25.5</td>
<td></td>
</tr>
</tbody>
</table>

In general the total size of fluctuations for the repeat unit, $\sigma_{\text{repeat}}$, show significantly lower values for models using paracrystalline structure factors than models using Caillé structure factors when performed on SAXS scattering data (Figures 3.11 and 3.16). This tendency can not be recognized in SANS, where $\sigma_{\text{repeat}}$ for the different models are comparable in size. The paracrystalline structure factor tends to underestimate $\sigma_{\text{repeat}}$ in order to fit the amplitude of the power law shaped diffraction peaks in SAXS, whereas the Caillé structure factor fits the shape of the peaks well. The opposite happens for SANS, where $\sigma_{\text{repeat}}$ is well estimated by the paracrystalline structure factor, which might be due to its ability to fit Gaussian shaped diffraction peaks.

### 3.5 General Conclusion on SANS and SAXS

Paracrystalline and Caillé theory take both the liquid crystalline nature of multi-lamellar lipid bilayer systems into account and by using these theories together with form factors derived by parameterization of the scattering length density profiles it is possible to derive expressions for the scattering functions. By fitting these scattering functions to small-angle neutron and x-ray scattering data it is possible to obtain structural information of the multi-lamellar bilayer system.

This kind of structural information is in a broader context very important for understanding the behavior of living cells whereas it is important to determine the theories and models that best describe the biological system.

Parameters fitted to SANS and SAXS are on an overall level not directly comparable, since SANS scattering data are obtained on nuclei and SAXS scattering data on electrons. This subsequently leads to different parameter values for SANS and SAXS e.g. illustrated by the thickness of the head group. For SANS the carbonyl-oxy group is a part of the acyl-chain, while it for SAXS is a part of the head group. However, by taking the chemical knowledge about lipids into account the structural characterization obtained by SANS and SAXS become very similar and comparable.

SANS is best at contrast matching, while SAXS has a better resolution. In relation

to structural characterization, SANS is preferable when e.g. placement of different subgroups of the bilayer is studied, while SAXS should be used when higher resolution of data is required.

Based on the comparative study of theories and models appropriate for structural characterization of multi-lamellar lipid bilayers it can be concluded that it is not obvious which theory and model to use, when performing fits to small-angle neutron and x-ray scattering spectra.

Paracrystalline theory with absolute widths of the layer thickness distribution obtains slightly but not significantly better fits than special Caillé theory when performing fits to SANS scattering data, while special and modified Caillé theory are more suitable when performing fits to SAXS scattering data. Special and modified Caillé theory can both be used with relative and absolute widths of the layer thickness distribution on SANS, while only with relative widths on SAXS.

For SANS it is not possible to determine whether the simple stochastic description underlaying paracrystalline theory is more appropriate than the harmonic description of the free energy density underlaying Caillé theory. It can however be concluded that in relation to the short-length scale the harmonic description is more adequate in describing the system for SANS. For SAXS the underlaying harmonic description of the free energy density in Caillé theory is also better in describing the bilayer system.

Furthermore it can be concluded that the physical assumption about fluctuations of the different parts of the multi-lamellar bilayer system being relative to the thickness of the respective parts is not valid for paracrystalline theory while valid for decoupled theories. This leads to the conclusion that the size of the water layer fluctuation is independent of the size of the fluctuations within the bilayer.

From the result of the fits, it can also be concluded that Caillé theory accounts for the diffuse scattering in the spectra, whereas paracrystalline theory requires an additional diffuse term, arising from the scattering of uncorrelated bilayers, to describe the bilayer system. It should also be noticed that neither paracrystalline nor Caillé theory should be applied to the spectra of the $P_{\beta'}$-phase, since they do not include the ripple structure of the bilayer in this phase.

A general conclusion on the best scattering length density profile model to use is not possible due to lack of resolution. The scattering length density profile 3GSM is recommended when performing fits to SANS spectra. Though, it is not possible to obtain stable fits with 4GSM and GM, which might be due to contrast matching by deuterium. For SAXS spectra no significant differences in fits performed with 3GSM and 4GSM are observed, though 4GSM obtains a slightly thicker hydrophobic core than 3GSM. We do however recommend to use 3GSM, due to the lower number of free structural parameters required. The GM model is also a suitable alternative to 3GSM when fitting MCT to SAXS spectra, since both models perform good fits and need the same number of free structural parameters.

Finally, we suggest to use SCT with 3GSM as an overall model, since SCT (3GSM) obtains fits of high quality when performing fits to both SANS and SAXS spectra. If looking for a model, which can be applied to other phases than the $L_{\alpha}$-phase, then PT (3GSM) with relative widths of the layer thickness distribution should be chosen, since PT is not limited to the $L_{\alpha}$-phase by its assumptions as it is the case with both
versions of the Caillé theory.
Part II

Structural and thermal characterization of ternary systems of di-myristoyl-phosphatidyl-choline, PEO-PPO-PEO tri-block copolymer (Pluronic® P85) and salt solution
Chapter 4

Introduction

Within the last decades there has been significant research in controlled drug delivery systems and drug carriers. These studies include soluble polymers, lipoproteins, microparticles made of insoluble or biodegradable natural and synthetic polymers, microcapsules, micelles and liposomes. Each of these carrier types offer their own advantages but has generally also serious disadvantages. Phospholipid liposomes are promising drug delivery systems, due to their biocompatibility and ability to entrap and transport hydrophilic species. Pure liposomes, on the other hand, show already after short-time use a highly reduced life-time in the body. A route to control and increase this liposome life-time in vivo may be to control the steric stability by incorporating surface active polymers into the system.

In the attempt to be able to design a specific drug delivery or transport system, it is essential to develop a better basic understanding of the physical-chemical effect that the surface active polymers might have on the thermodynamic properties of the lipid bilayer systems. There has already been some reports concerning liposomes incorporated with the poly(ethylene oxide) type of copolymers, but with somewhat diverging conclusions (Baekmark et al., 1997; Kostarelos, Kipps, F.Tadros and Luckham, 1998; Johnsson et al., 1999; Bryskhe et al., 2001; Torchilin, 2001; Chandaroy et al., 2002; Johnsson and Edwards, 2003). There is therefore a need for further systematic studies of such systems.

This part of the ph.d. thesis will be addressed to structural and thermal characterization of the DMPC/P85 system, which can serve as a drug delivery system (Jamshaid et al., 1988; Moghimi et al., 1991).

4.1 Drug delivery systems

In the broadest sense, a drug delivery system is a way to give a drug, and the most common systems are pills, soluble tablets and injections. There is however a drawback with these kinds of delivery systems; they expose the whole body to the drug. This is why drug targeting or site-specific drug delivery systems have been developed, cause they improve the traditional delivery systems by:

- reducing the exposure of the active drug(s) to other tissues.
• reducing the total dose of the active drug(s).
• making active drugs available for therapeutic use, which for the time being are not accepted because of their toxicological properties.

4.1.1 Drug Targeting

Delivering of a drug to a specific tissue of interest at an appropriate concentration is the main purpose of drug targeting, cause in that way the body is protected from the unwanted side effects of the drug.

![Figure 4.1: The figure shows the principle of site-specific drug carriers.](image)

Site-specific drug delivery or drug targeting is already a common used method. Just think of all the asthmatic patients who use an inhaler containing drugs against asthma, or the use of disinfection agents to prevent infections.

This kind of drug targeting is straight forward, since the asthma inhalers or the disinfection agents affect the external epithelial tissue. However, if the pathological site is less accessible, the site-specific drug delivery system needs an additional property, namely a drug carrier, which makes the pathological tissue accessible in a site-specific manner. A drug carrier encapsulates the drug and avoids thereby the unfavorable contact between body tissue and the active drug(s).

Many different components or micro-particles have been suggested to serve as drug carriers (Langer, 1998; Pouton, 1997; Mrsny, 1997; Duncan, 1997). Thus, an ideal drug carrier should both be able to limit unwanted side effects and degradation of the active drug(s) as well as release the drug(s) within the appropriate time frame, in the wanted form and phase. If a drug is insoluble in an aqueous solution, then the main task of the drug carrier is keeping the drug dissolved until the target site is reached. When the drug carrier has no more function it should ideally be biodegradable or at least easy to remove from the body, as it may not be toxic or immunogenic. Finally, the ideal drug carrier should be cheap and stable through storage.

Some of the systems which have served as drug carriers, include liposomes (Gregoriadis and Ryman, 1971; Gregoriadis, 1973), surfactant micelles (Trubetskoy and Torchilin, 1995), nanoparticles (Kreuter, 1991), cells and virus (Blumenthal and Loyter, 1991).
4.1.2 Liposome drug carriers

Sessa and Weissman (Sessa and Weissman, 1968) showed more than 30 years ago, that soluble components can be entrapped in the aqueous compartment of the liposomes, and later on in the early 1970’s Gregoriadis et. al. suggested that liposomes could serve as drug and enzyme carriers (Gregoriadis and Ryman, 1971; Gregoriadis, 1973).

In vivo experiments show that intravenously injected liposomes into animals are quickly removed from the bloodstream by the MPS-cells (phagocytic cells of the mononuclear phagocyte system), especially in the liver and spleen (Illum et al., 1982; Poste and Kirsh, 1983). This is a reaction of the body’s immune system to foreign material, cause when the MPS-cells recognize proteins adsorbed to the liposome surface, they will take them up and remove them from the body, most likely through the liver and spleen.

To prolong the lifetime of the liposomes in the bloodstream, different soluble polymers have been used to coat the outer surface of the liposomes, e.g. poly(ethylene glycol), also denoted PEG (Klibanov et al., 1990; Senior et al., 1991; Allen et al., 1991; Papahadjopoulos et al., 1991). Due to the helical structure of PEG oxygen in the backbone is able to make water bridges with the adjacent monomers (Begum and Matsuura, 1997; Heymann and Grubmüller, 1999). This water shield makes the PEG chains look like water, whereas the proteins will not be able to penetrate the shield and thereby make contact and adhering to the PEG-chains. If the density of PEG grafted to the liposome surface is high enough, then the PEG layer will sterically hinder the proteins from adhering to the liposome surface (Figure 4.2). Such steric stabilized liposomes or Stealth® (a trademark of Liposome Technology, Inc. Menlo Park, CA, USA) have been shown to circulate longer in the bloodstream (Klibanov et al., 1990), and being a more efficient drug carrier to target tissues.

![Figure 4.2](image-url) Figure 4.2: The figure shows the principle of steric stabilized liposomes. In (a) the liposome is not steric stabilized, whereas the protein is able to adhere to the surface of the liposome. In (b) the liposome is steric stabilized, e.g. with PEG grafted to the liposome surface, whereas the protein is not able to make contact to the liposome and will be swept away.

The dependencies between the lifetime of PEG-lipids and the length of the hydrophobic acyl-chain are shown by Holland et al. (Holland et al., 1996). For short acyl-chain PEG-lipids, the surface density of the liposomes gradually decreases during blood circulation and causes a loss in the steric stability. As shown in Figure 4.3 triblock copolymers of the PEO-PPO-PEO type, e.g. Pluronic®, can either be absorbed
(Jamshaid et al., 1988; Moghimi et al., 1991) or incorporated into the bilayer during the liposome formation (Kostarelos, Kipps, F.Tadros and Luckham, 1998; Kostarelos, Luckham and F.Tadros, 1998; Kostarelos et al., 1999).

Figure 4.3: The figure shows different inclusion modes for the Pluronic® in the liposomes. (a) shows a configuration with both PEO chains on the same side, while (b) shows the membrane spanning configuration.

In recent years, the interactions between PEO-PPO-PEO tri-block copolymers and phosphatidylcholine liposomes have been investigated by Kostarelos et al. (Kostarelos, Kipps, F.Tadros and Luckham, 1998; Kostarelos, Luckham and F.Tadros, 1998; Kostarelos et al., 1999; Johnsson et al., 1999). They have also studied how different kinds of incorporations affect the degree of steric stabilization of the liposomes with both dynamic light scattering (Kostarelos, Luckham and F.Tadros, 1998; Kostarelos et al., 1999) and NMR (Kostarelos, Kipps, F.Tadros and Luckham, 1998; Kostarelos et al., 1999). The studies showed, that if copolymer participates in the liposome formation, and thereby able to span the whole bilayer, then the stabilization effect upon the liposome is better than if they were just adsorbed to the bilayer.

Chandaroy et al. have also studied the effect of adhesion between cells and liposomes incorporated with PEO-PPO-PEO type of block copolymers (Chandaroy et al., 2002) and they found a major temperature dependency assumed to be associated with the block copolymer micelle formation. Johnsson et al. have in other studies used cryo-transmission electron microscopy and dynamic light scattering to characterize the aggregate structure and phase behavior of mixtures of PEG-lipids, with molecular weights of 2000 or 5000 of the PEG component, and distearoylphosphatidylcholine and dipalmitoylphosphatidylcholine respectively (Johnsson et al., 1999; Johnsson and Edwards, 2003). Johnsson et al. found in their study a transition from a dispersed lamellar liposome-phase to a micellar-phase, with micelle formation starting at PEG-lipid concentrations less than 5 mol%.

Landh has in a study of aqueous systems of single-chained lipid-monoglyceride shown miscibility with EO$_{99}$PO$_{67}$EO$_{99}$ triblock copolymers (Poloxamer 407) (Landh,
1994). By using small-angle x-ray scattering Landh revealed four different reversed bilayer-based cubic bicontinuous phases: the Ia\(^{3d}\) gyroid phase (G), the Pn\(^{3m}\) double diamond phase (D), the Schwarz primitive structure (P), and the Neovius periodic minimal surface structure (C) (Landh, 1994). At concentrations higher than 20 wt% poloxamer 407 the cubic P-phase is transformed to a reversed micellar (L\(_2\)) phase. The phase diagram possesses two lamellar (L\(_\alpha\)) phases, of which Landh suggested one to originate from the monoglyceride-water system. Funari et al. (1997) found related structural characteristics in the POPE-C\(_{12}\)EO\(_2\), system with POPE being the abbreviation of phosphatidylethanolamine, and by using x-ray scattering, they found a L\(_\alpha\)-phase at 20\(^\circ\)C, a Ia\(^{3d}\) gyroid phase at intermediate temperature and a Pn\(^{3m}\) double diamond phase at 39\(^\circ\)C.

In a x-ray and NMR study by Oradd et al., it has also been shown that soybean phosphatidylcholine together with diacylglycerol and water forms either lamellar, reversed hexagonal or cubic phases, depending on the concentration and temperature (Oradd et al., 1995). This extension of the region of the cubic phase do however not seem to change the appreciably for the ternary system, which could be due to the cubic phase being built up by closed packed reversed micelles.

Sadaghiani et al. have in a pulsed NMR study, shown that solutions of poly(ethylene oxide)-octylphenylether (abbreviated Triton X-100) can solubilize lecithin up to 10 wt% (Sadaghiani et al., 1989; Sadaghiani and Khan, 1991). Sadaghiani et al. found in the same study micelles or lamellar phases, depending on the composition. Khan et al. have also studied the solubilization of water-soluble poly/(ethylene oxide) homopolymer into the lamellar phase of soybean lecithin, again by using NMR and polarizing microscopy methods (Khan et al., 1994). Their study reveals that between 20 and 33 wt% of water, the liquid crystalline lamellar phase is unable to solubilize any appreciable amount of polymers and that the mesophase is destabilized by the addition of about 0.5% of polymers, whereas the solubilization of polymers increases drastically in the lamellar phase with a water content less than 10%. The results indicate that the binding of water to the interface and the molecular organization of the choline head group are little affected by the presence of polymer molecules in the mesophase.

Baekmark et al. have studied unilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) mixed with different poly(ethylene oxide) based block copolymers: DSPE-EO\(_{45}\), DSPE-EO\(_{110}\) and EO\(_{42}\)S\(_{12}\)EO\(_{42}\), where DSPE is the abbreviation of distearoylphosphatidylethanolamine, and S\(_{12}\) is polystyrene (Baekmark et al., 1997). Their study showed that the main phase transition temperature changes only few \(^\circ\)C upon addition of polymers, but also that an additional enthalpy peak can be observed at temperatures slightly above the main transition.

Finally, Bryskhe et al. have made a rather systematic study on soybean phosphatidylcholine and EO\(_4\)PO\(_8\)EO\(_5\) copolymers (abbreviated Pluronic\(^\circ\) L121) in aqueous mixtures, focusing on the ambient temperature phase behavior (Bryskhe et al., 2001). The used PEO-PPO-PEO block copolymer is in itself bilayer-forming at ambient temperatures and Bryskhe et al. found that the block copolymer and lecithin are immiscible.

In the present ph.d. thesis, the ternary system of P85 (EO\(_{25}\)PO\(_{40}\)EO\(_{25}\)), DPMC and salt solution has been studied.
4.2 P85 triblock copolymer

P85 is an amphiphilic block copolymer, and belongs to the Pluronic® family. A block copolymer is composed of blocks or sequences of polymers, which are chemically distinct. The most simple class of block copolymer is the di-block copolymer, which consists of two blocks (A-B). Another class is tri-block copolymer of the type A-B-A, which Pluronic® belongs to. By varying the composition of the block copolymer and the length of the individual segments, a number of copolymers exhibiting a huge range of physical and chemical properties are obtained.

To be more specific, the Pluronic® belongs to the PEO-PPO-PEO tri-block copolymer family, and contains a sequence of poly(ethylene oxide) and poly(propylene oxide).

\[
\text{HO} - \text{[CH}_2\text{CH}_2\text{O}][\text{m}] - \text{[CH}_2\text{CHO}][n] - \text{[CH}_2\text{CH}_2\text{O}][m] - \text{H}
\]

Figure 4.4: The figure shows the chemical structure of Pluronic®

When the molecular weight of PPO goes beyond 740 g/mol, it changes from being water soluble to water insoluble. The solubility of PPO and PEO also depends on the temperature; PPO is hydrophobic above app. 15°C, whereas PEO does not change from hydrophilic to hydrophobic until app. 70°C. Pluronic® is therefore amphiphilic in the temperature range from app. 15 to 70°C.

Pluronic® is a registered trademark within the BASF Corporation, but PEO-PPO-PEO tri-block tri-block copolymer is also available under other commercial names such as Poloxamers. Pluronic® has extensively been studied, because of the great potential for commercial application, i.e. lubrication and surfactant biological application (Ref. Alexandridis and Hatton (1995); Spontak and Alexandridis (1999); Yang and Alexandridis (2000)).

4.2.1 Pluronic® Grid

To get an overview of the relationship between the physical and chemical properties versus the different ratios and weights of the Pluronic® family, a graphical representation is shown in figure 4.5. The nomenclature for the Pluronic® family consists of a letter followed by two or three digits, where the letter indicates the phase of the Pluronic® at 20°C, 'L' is for liquid, 'P' for paste and 'F' for flake (the solid lamellar form). The first one or two digits indicate the app. weight of PPO in units of 300g/mol, while the last digit multiplied by 10 indicates the total content of PEO in wt%.

4.2.2 Phase diagram

The structure of bulk P85 in aqueous solution is determined as a function of concentration, temperature and pressure. Various complementary techniques have been used in the determination, e.g. small-angle neutron and x-ray scattering, dynamic
4.2. P85 TRIBLOCK COPOLYMER

Figure 4.5: The figure shows a plot of the molecular weight of the hydrophobic poly(propylene oxide) part against the total weight-percentage of poly(ethylene oxide) in each molecule. The plot points out the relationship between the structure and physical properties. The fraction of PEO has a high effect on the phase of the Pluronic® at $T = 20^\circ C$. The Pluronic® goes from being liquid at low fraction (dark shade area), to become paste in the intermediate range (white area) and finally being flakes at high fraction (light shade area). The plots is adapted from the BASF Corporation (BASF Corporation, 2001).

and static light scattering, differential scanning calorimetric (DSC) etc. (Brown et al., 1991b; Glatter et al., 1994; Mortensen and Pedersen, 1993). A phase diagram showing temperature versus polymer concentration is illustrated in Figure 4.6.

The tri-block copolymer is completely dissolved and exists as unimers in the low temperature and concentration region of the phase space. Mortensen and Pedersen determined the radius of gyration, $R_g$, to 17 Å (Mortensen and Pedersen, 1993). The Gaussian chain statistic predicts however a slightly larger value according to $R_g^2 = bL/6$, where $b$ is the statistical segment (Kuhn) length and $L$ is the contour length. Mortensen furthermore suggested that PPO could be more dense than expected from a Gaussian chain (Mortensen, 1996).

As the temperature and concentration increases, the copolymer molecules begin to self-assemble in small aggregates of the micelle type. The aggregation takes place when either the temperature reaches the critical micelle temperature (CMT) or the concentration reaches the critical micelle concentration (CMC). Alexandridis et al. (Alexandridis et al., 1994) have also reported a large compilation of CMT and CMC for Pluronic® tri-block copolymer, when using a dye stabilization method. As for lipids, the aggregation of Pluronic® tri-block copolymer is forced by the hydrophobic effect (Tanford, 1980).

P85 forms spherical micelles in the temperature range of 30 to $70^\circ C$ and with a copolymer concentration between 0 and 25 wt%. The micelles have a dynamic structure, where the copolymers in the micelles are in a dynamic equilibrium with the
copolymers in solution. The lifetime of P85 molecules in micelles have been studied with field gradient NMR by Fleischer (Fleischer, 1993), and the experiment showed that each copolymer has a lifetime of 1 µs to 1 ms, at concentrations below 10 wt%. From SANS measurements Mortensen and Pedersen (Mortensen and Pedersen, 1993) have furthermore showed that the size of the micelles depend more on the temperature than the copolymer concentration. This is in accordance with ones expectations, since EO binds less water per monomer as the temperature increases.

![Figure 4.6:](image)

Figure 4.6: The figure shows the phase diagram of a P85 water binary system, with temperature versus concentration. Adapted from Mortensen (Mortensen, 1996)

The micelles undergo a phase transition when the temperature increases above app. 70°C, which brings them into a regime with a rod-like structure (Mortensen and Pedersen, 1993). The radius of the core is determined to 50 Å, which gives an average of only
2.5 Å per PO monomer for chains going through the center of the micelles (Mortensen and Pedersen, 1993). When the temperature increases above 70°C, EO looses its water, which makes it possible for EO to mix with PO in the center of the micelles. This is also indicated in the lattice model calculation by Noolandi et al. (Noolandi et al., 1996), where micelles arrange themselves in a BCC lattice at concentrations above app. 25 wt%. The transition into a BCC liquid-crystalline phase is a first order transition, and the viscosity of the system increases dramatically throughout the transition. When the temperature and polymer concentration increases furthermore a new phase transition appears with P85 aggregating into rods, who arrange themselves in a hexagonal lattice.

Finally, it should be noticed that at low water content and temperature P85 will crystallize in a lamellar structure of alternating sheets of high stretch PPO and PEO (Mortensen, 1996). For temperatures above the melting point of PEO, a disordered phase can be observed. Regarding P85, the tri-block copolymer keeps its lamellar structure up to 50 wt% water content, whereas above the PEO melting point P85 forms a PPO lamellar structure (Mortensen, 1996).
Chapter 5

Experimental section

This chapter contains description, analysis and discussion of experimental calorimetric and structural studies of aqueous systems of dimyristoyl-phosphatidylcholine lipids (DMPC) incorporated with PEO-PPO-PEO type of block copolymers. The studies include both variations in polymer to lipid ratio, content of water and temperature.

The P85 content of the samples, is defined by,

$$w_{P85} = \frac{m_{P85}}{m_{P85} + m_{lipid}} \cdot 100\%$$

and is measured in units of wt%.

5.1 Materials and Methods

5.1.1 Materials

Dimyristoyl-phosphatidylcholine, DMPC, with a purity higher than 99% is purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) tri-block copolymer, Pluronic® P85, with mean composition (EO\textsubscript{27}-PO\textsubscript{38}-EO\textsubscript{27}) is a gift from the BASF Corporation. The poly(ethylene glycol) homopolymers, PEG1000 (~EO\textsubscript{23}) and PEG4000 (~EO\textsubscript{91}), are gifts from Hoecht AG. Other chemicals are purchased from Sigma Chemical Co, all used without further purification.

5.1.2 Methods

The lipid mixtures are made by mixing DMPC with P85 or PEG in a solvent consisting of an equal amount of chloroform and methanol. After being mixed the solvent is evaporated under a clean dry Nitrogen stream, and subsequently dried under vacuum in order to remove traces of solvent and water for at least 12 hours. Multilamellar bilayers are created by hydrating the lipid mixture in a desired amount of aqueous salt solution [50 mM KCl and 1 mM NaN\textsubscript{3}] for a minimum of 2 hours at $T > T_m + 10^\circ C$, which is about $10^\circ C$ above the main-melting-transition, $T_m \approx 25^\circ C$, of DMPC. The aqueous salt solution are in the following abbreviated solvent or water, for simplicity.
During the hydration the samples are shaken vigorously with a vortex-mixer and cycled through $T_m$ every 15 minute.

The samples are defined in terms of water content and polymer concentration, with the latter defined as $w_{P85} = m_{P85}/(m_{P85} + m_{DMPC})$, where $m_{P85}$ and $m_{DMPC}$ are the total masses of respectively P85 block copolymers and DMPC lipids.

The equilibrium for samples with a low DMPC/P85 concentration is checked by liquid differential scanning calorimetry carried out on a Microcal MC-2 DSC instrument. It is however not possible to use liquid differential scanning calorimetry on samples with high DMPC/P85 concentration. Thus, viscous samples can not be transferred into the sample well and the high DMPC/P85 concentration will also causes an overflow. To ensure the samples with high DMPC/P85 concentrations have reach equilibrium, they are stored at room temperature for at least two weeks.

In the studies comparing the influence of P85 and PEG, the molar amount of PEG1000/4000 equals twice the molar amount of P85, which corresponds to equal molar amount of the PEO-chain in P85 (Figure 5.13).

Figure 5.1: The figure shows the SAXS camera from which the spectra are obtained.

The SAXS measurements are performed on the SAXS camera placed at Risø National Laboratory, which is equipped with a two-dimensional gas detector (Gabriel EMBL, France), operating at a voltage of 3.94 kV and with a working potential of 1.5 kV. The camera is connected to a rotating cobber anode x-ray generator from Rigaku, Japan, working at a voltage of 50 kV and a current of 300 mA and filtered by a pyrolytic graphite monochromater crystal. A schematic illustration of the SAXS camera is shown in Figure 5.2.

The SAXS measurements are carried out with a wavelength of 1.542 Å and a sample-to-detector distance of $\sim$1 m, which gives a $q$-range from $\sim$0.02 - 0.5 Å$^{-1}$. The collimation of the beam is determined by three sets of slits; one just after the source and two close to the sample. The slits give a square shaped beam with a width of 1 mm at the sample position. If nothing else is mentioned the x-ray acquisition time is typically 2 hours at three different temperatures, 10, 20 and 35°C. The sample temperature is regulated by a home made oven designed by Finn Saxild at Risø National Laboratory. The oven is controlled from the computer through a thyristor temperature control unit.

All samples used in the small-angle experiments are transferred to 2 mm $\varnothing$ borosilicat capillary tubs (W. Müller, Germany) sealed with wax.
5.1. MATERIALS AND METHODS

Figure 5.2: The figure shows a schematic illustration of the SAXS set-up. The figure only shows the slits determining the vertical position and size of the x-ray beam.

Figure 5.3: The figure shows the oven used to control the temperature of the different samples.

The DSC measurements of the high concentration samples are carried out by using a Perkin-Elmer Pyris 1 DSC, to which ~20 mg of the samples are transferred on small aluminium wells and sealed. After equilibrated for 12 hours at 5°C, each sample is scanned successive two times, with scan rates of 1°C/min and 2°C/min respectively. The samples are equilibrated at 5°C for 50 minutes between the two successive scans.
5.2 DMPC Mixed with P85

In this section results obtained from studies of the ternary DMPC/P85/solvent system are being presented. First, the binary DMPC/solvent system is briefly being treated; secondly data output from the SAXS measurements are being analyzed and discussed.

It should be noticed that the ratio between DMPC and P85 and the total concentration of DMPC and P85 has been changed during the experiments.

5.2.1 Binary DMPC/solvent System

SAXS spectra of DMPC/solvent systems obtained at 20°C, show that the \((0,n)\) ripple peaks increase in intensity as the solvent content decreases and the DMPC concentration increases (Figure 5.4a). The spectra also show that the ratio of the first order ripple peak and the first order lamellar peak is constant and within experimental accuracy. From Figure 5.4a it can furthermore be seen, that the shoulder on the high-\(q\) side of the \((1,0)\) and \((2,0)\) order diffraction peaks is designated to the respectively \((1,1)\) and \((2,1)\) ripple peaks and that the growth in amplitude is caused by an increasing DMPC concentration. In overall, the intensity of the different peaks follows the increase in the DMPC concentration.

![Figure 5.4: The figure shows SAXS spectra obtained on samples of pure DMPC with various solvent contents.](image)

At 35°C, the intensity of the lamellar peaks show the same development as for 20°C (Figure 5.4b). The peak positions are however not affected by the change in the DMPC concentration as long as the solvent content is above 40 wt%. At 30 wt%, the peak positions shift towards higher \(q\)-values which indicates the repeat distance is decreasing. The visibility of the third order diffraction peak also indicates that a higher degree of order exists within DMPC/solvent systems with solvent contents below 40 wt%.
The existence of third order diffraction peaks and decreasing repeat distance is in accordance with the behavior of not-fully hydrated systems (Figure 1.6a). From Figure 1.6a it can also be seen that the limit of excess water changes at the main-phase-transition, and that the limit is around 30 wt% below and 40 wt% above the main-phase-transition.

5.2.2 Ternary DMPC/P85/solvent System

Scattering data of the ternary DMPC/P85/solvent system, obtained at 20 and 35°C, are shown in Figure 5.5 and 5.6.

Observations

Low-temperature gel-phase The SAXS spectra begin to undergo changes even when small amounts of P85 wt% are being added to the DMPC/solvent mixture. At \( w_{\text{P85}} = 0.1 \) wt% and a solvent content = 30 wt%, two additional peaks can be observed between the second and third order diffraction peaks, and as the weight fraction of P85 increases more features become visible in the spectra. At \( w_{\text{P85}} = 2 \) wt%, the shoulder on the high-\( q \) side of the first order diffraction peak is more pronounced, while the peak at around 0.2 Å\(^{-1}\) begins to split into two on the low-\( q \) side. This remains more or less until \( w_{\text{P85}} = 90 \) wt%, above this level the peaks begin to disappear. At \( w_{\text{P85}} = 3 \) wt%, a shoulder becomes visible on the low-\( q \) side of the large peak located around 0.1 Å\(^{-1}\). As the P85 weight fraction increases the shoulder splits into a separate intense peak, though it is not as intense as the 0.1 Å\(^{-1}\) peak.

The SAXS spectra also undergo tremendous changes at solvent contents higher than 30 wt%. At 53 and 70 wt%, the spectra changes completely at around \( w_{\text{P85}} = 3 \) wt%. Thus, first order ripple peaks are disappearing while new ones start occurring for \( q \geq 0.1 \) Å\(^{-1}\). These peaks are sharper than the peaks observed at \( w_{\text{P85}} \) below 3 wt%. As the P85 weight fraction increases, the spectra for samples with higher solvent contents begin to change. At \( w_{\text{P85}} = 10 \) wt%, the change can also observed for samples with a solvent content up to 97.5 wt%.

A further increase in the P85 weight fraction causes another dramatic change in the spectra, thus the spectra begin to become more and more similar to the ones obtained at a solvent content of 30 wt%. Again, the changes are first observed for a low solvent content = 53 wt% and a P85 weight fraction of 40 wt%, and they last until the solvent content = 97.5 wt% and \( w_{\text{P85}} = 90 \) wt%.

By comparing all SAXS spectra obtained on samples with a P85 weight fraction between 20 and 90 wt% it can be seen, that only spectra with solvent contents of 30 and 53 wt% have peaks occurring in the \( q \)-range around 0.05 Å\(^{-1}\).

At \( w_{\text{P85}} = 99 \) wt% and above, changes can again be observed in the SAXS spectra (Figure 5.5f). By splitting the spectra into two groups, with the first group consisting of spectra obtained on solvent contents up to \( \sim 80 \) wt%, it can be seen that the shape, relative height and position of the peaks are more or less the same for the first group. The peaks occur at 0.07 and 0.11 Å\(^{-1}\) for all spectra up to \( \sim 80 \) wt% and furthermore at 0.13 and 0.23 Å\(^{-1}\) for spectra at 30 wt%. The second group, which consists of
Figure 5.5: The figure shows the SAXS spectra obtained on samples with various P85 weight fractions and solvent contents, at 20°C.
Figure 5.6: The figure shows the SAXS spectra obtained on samples with various P85 weight fractions and solvent contents, at 35°C.
spectra obtained on solvent contents above $\sim 80$ wt%, has the same background as the first group, though the peaks from the first group can not be recognized here. The spectrum of 85 wt% solvent content has also two small peaks at around 0.1 and 0.2 Å$^{-1}$ which can be distinct from the background, whereas no such peaks can be seen in the spectrum of 97.5 wt%.

Observations

High-temperature lamellar-phase For spectra obtained at 35$^\circ$C, the only effect of increasing P85 up to $w_{\text{P85}} = 2$ wt% is an enhancement of the intensity between the first and second order peaks, when compared to the pure DMPC system. Again, spectra obtained on samples with a 30 wt% solvent content exhibit a different behavior, which is due to the not-fully hydrated system, whereas only minor effects of P85 can be observed. At $w_{\text{P85}} = 2$-3 wt% and a solvent content between 53 and 97.5 wt%, the spectra changes dramatically. Especially the spectra of 53, 70 and 85 wt% solvent undergo tremendous changes, with a broad intense peak appearing between the first and second order diffraction peaks. The broad peak is most pronounced for lower solvent contents, and as the P85 weight fraction increases the peak becomes more sharp. A similar peak can be observed in the spectrum of 97.5 wt% solvent, thus here it is observed for higher levels of P85 and has a very smeared peak shape. In fact, the scattered intensity of this sample appears more as a very broad peak than three distinct peaks.

For $w_{\text{P85}} = 10$ wt% and a solvent content of 30 wt%, an additional peak can be observed at 0.072 Å$^{-1}$, and the intensity of this peak increases with increasing fraction of P85. No other changes are observed in the spectra until $w_{\text{P85}} = 80$ wt%, where all peaks become more sharp. For $w_{\text{P85}} = 80$ wt%, new shoulders/peaks appear on the high-q side of the two peaks positioned at 0.071 and 0.124 Å$^{-1}$, furthermore the peaks positioned at 0.12, 0.24 and 0.35 Å$^{-1}$ shift towards lower q-values as the P85 weight fraction increases. The shoulder/peak on the high-q side of 0.12 Å$^{-1}$ and the peak at 0.07 Å$^{-1}$ grow to be intense peaks, while the other peaks disappear from the spectra. The spectra obtained at 35$^\circ$C, with a P85 weight fraction $\geq 80$ wt% and solvent content = 30 wt%, thereby exhibit the same behavior as the ones obtained at 20$^\circ$C. Equivalent similarity can be observed in the spectra obtained at $w_{\text{P85}} \geq 99$ wt%.

At $w_{\text{P85}} \geq 40$ wt%, the spectra performed on samples containing 53 wt% solvent are similar to the ones obtained at 30 wt% solvent. For solvent contents of 70 and 85 wt%, the spectra do not change until the P85 weight fraction is at least 70 wt%. Above this level the spectra changes and become similar to the ones obtained at 20$^\circ$C, as the P85 weight fraction increases.

Discussion

Low-temperature gel-phase For SAXS spectra obtained at 20$^\circ$C, a tremendous change occurs at $w_{\text{P85}} = 3$ - 10 wt% and a solvent content $> 30$ wt%. A plausible hypothesis for this change can be the occurrence of a phase transition when the P85 weight fraction increases above 3 wt%. From the peak positions in Figure 5.7 it can
furthermore be seen that the lamellar structure of the pure DMPC system disappears for P85 weight fractions = 10 wt% and solvent contents of 53, 70, 85 and 97.5 wt%. Other possible structures of DMPC can not be seen in the phase diagram (Figure 1.6a).

Figure 5.7: The figure shows peak positions for spectra obtained at \( w_{\text{P85}} = 10 \) wt% and solvent contents of 53, 70, 85 and 97.5 wt%. The peak positions are found by fitting the Lorentzian peaks to the spectra, corrected for the powder averaging by multiplying \( q^2 \). Notice, that peak positions for the pure DMPC system at 20°C have been added to the figure for comparison.

From the phase diagram of P85 (Figure 4.6), it can be seen that P85 arranges in both a hexagonal \((H_1)\) phase and BCC \((\text{Im\bar{3}m})\) liquid crystalline lattice, consisting of closed packed micelles depending on the composition. In Table 5.1 some of the allowed Miller indices are listed for the two structures.

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</tr>
</tbody>
</table>

Table 5.1: The table shows some of the allowed Miller indices for the Hexagonal \((H_1)\) and BCC \((\text{Im\bar{3}m})\) structures. The indices are listed together with the squared length of the scattering vector, which is given in units of the squared reciprocal lattice parameter.

In the attempt to determine the structure of the DMPC/P85 mixture with \( w_{\text{P85}} = 10 \) wt% and solvent contents of 53, 70, 85 and 97.5 wt%, we have tried to index the spectra as either a Hexagonal \((H_1)\) or BCC \((\text{Im\bar{3}m})\) structure. From Table 5.2 it can however be seen that neither Hexagonal nor BCC are plausible structures for the DMPC/P85 mixture, since the indexation is not in accordance with the allowed peaks.

Funari et al. (Funari et al., 1997) have in x-ray and NMR studies of the POPE-C12-EO2 system shown that a sample composition of 46.3 wt% H2O, 46 wt% \( C_{12}\text{EO}_2 \) and
CHAPTER 5. EXPERIMENTAL SECTION

Hexagonal (H1) phase

<table>
<thead>
<tr>
<th>a</th>
<th>h + k + l^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04306</td>
<td>4.66 6.00 8.57 11.82 15.86 18.70 23.64 24.94 36.89</td>
</tr>
<tr>
<td>0.04421</td>
<td>3.34 4.00 5.13 7.49 10.45 15.60 17.35 20.87 24.22</td>
</tr>
<tr>
<td>0.03956</td>
<td>4.63 6.00 9.36 12.74 15.65 18.89 25.87 35.94 61.18</td>
</tr>
<tr>
<td>0.03862</td>
<td>4.62 6.00 8.97 12.10 17.15 20.08 25.59 37.57 58.00</td>
</tr>
</tbody>
</table>

BCC (Im3m) phase

<table>
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<tr>
<th>a</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0.05365</td>
<td>3.00 3.87 5.52 7.61 10.21 12.05 15.23 16.06 23.76</td>
</tr>
<tr>
<td>0.04665</td>
<td>3.00 3.59 4.60 6.73 9.38 14.01 15.58 18.74 21.75</td>
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<tr>
<td>0.04913</td>
<td>3.00 3.89 6.07 8.25 10.14 12.24 16.76 23.30 39.65</td>
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<td>0.04790</td>
<td>3.00 3.90 5.83 7.86 11.15 13.05 16.63 24.42 37.70</td>
</tr>
</tbody>
</table>

Table 5.2: The table shows the best results from indexing the peaks to either the Hexagonal (H1) or the BCC (Im3m) structure. The values are the squared length of the scattering vector (h^2+k^2+l^2), and with the lattice parameter a shown in the first column.

7.6 wt% POPE undergoes several phase transitions; going from the lamellar Lα-phase at 20°C, through the Ia3d phase and ending in the Pn3m phase at 39°C. Both Ia3d and Pn3m are bicontinuous cubic phases, where Ia3d is of the gyroid prime type and Pn3m of the double diamond type. The index of allowed lower order Miller indices for both structures are shown in Table 5.3.

Cubic (Ia3d) phase

<table>
<thead>
<tr>
<th>(hkl)</th>
<th>h^2+k^2+l^2</th>
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<tr>
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<td>6 8 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
</tr>
<tr>
<td>220</td>
<td>6 8 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
</tr>
<tr>
<td>222</td>
<td>6 8 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
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<tr>
<td>311</td>
<td>6 8 10 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
</tr>
<tr>
<td>411</td>
<td>6 8 10 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
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<td>420</td>
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<td>521</td>
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<tr>
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<td>6 8 10 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
</tr>
<tr>
<td>544</td>
<td>6 8 10 12 14 16 18 20 22 24 26 30 32 34 36 38 42</td>
</tr>
</tbody>
</table>

Cubic (Pn3m) phase

<table>
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<tr>
<th>(hkl)</th>
<th>h^2+k^2+l^2</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2 3 4 6 8 9 10 11 12 14 16 17 18 19 20</td>
</tr>
<tr>
<td>532</td>
<td>2 3 4 6 8 9 10 11 12 14 16 17 18 19 20</td>
</tr>
<tr>
<td>544</td>
<td>2 3 4 6 8 9 10 11 12 14 16 17 18 19 20</td>
</tr>
</tbody>
</table>

Table 5.3: The table lists some of the allowed Miller indices for the bicontinuous cubic Ia3d and Pn3m phases. The indices are listed together with the squared length of the scattering vector, which is given in units of the squared reciprocal lattice parameter.

Again, we have tried to index the obtained spectra as either a Ia3d or Pn3m structure, and from Table 5.4 it can be seen that the structure can be identified as Pn3m. The Pn3m cubic phase is observed at low temperature in a rhombic shape like regime for copolymer concentrations between wP8 3 - 90 wt% and water concentrations >30 wt%. By comparing the samples it can furthermore be seen, that an increase in the solvent content from 53 to 97.5 wt% leads to an increase in the repeat distance of ~14 Å, going from 119.1 to 132.8 Å (Table 5.5). This is in clear contrast to the observations for the pure DMPC/solvent system, where the repeat distance only shows small fluctuations in the Pβ and Lα-phases, going from 64.5 to 66.7 Å in the Pβ-phase and from 61.6 to 64.5 Å in the Lα-phase. The general effect of the main-phase-transition can furthermore be observed as a decrease in the lattice spacing, when going from the Pβ to the Lα-phase.
5.2. DMPC MIXED WITH P85

Table 5.4: The table shows the best results from indexing the peaks to either the Ia $\bar{3}d$ or Pn $\bar{3}m$ structures. The values are the squared length of the scattering vector ($h^2 + k^2 + l^2$), and with the lattice parameter $a$ shown in the first column.

<table>
<thead>
<tr>
<th>Cubic (Ia $\bar{3}d$) phase</th>
<th>$h^2 + k^2 + l^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$</td>
<td>$h^2 + k^2 + l^2$</td>
</tr>
<tr>
<td>0.03794</td>
<td>5.00 7.73 11.04</td>
</tr>
<tr>
<td>0.03609</td>
<td>5.01 6.00 7.69</td>
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<tr>
<td>0.03474</td>
<td>6.00 7.78 12.13</td>
</tr>
<tr>
<td>0.03387</td>
<td>6.00 7.80 11.66</td>
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</table>

Table 5.5: The table shows the unit cell length, $d_{100}(Pn\bar{3}m)$, for samples with $w_{P85} = 10$ wt% and solvent contents of 53, 70, 85 and 97.5 wt%. The values are listed together with the repeat distance for both the $P_{\beta'}$, $d_{001}(P_{\beta'})$, and the $L_{\alpha}$, $d_{001}(L_{\alpha})$, phases of the pure DMPC system. The $a$’s are the respective reciprocal unit cell parameter.

<table>
<thead>
<tr>
<th>20°C (10%)</th>
<th>20°C (0%)</th>
<th>35°C (0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_{Pn\bar{3}m}$</td>
<td>$d_{111}$</td>
<td>$a_{P_{\beta'}}$</td>
</tr>
<tr>
<td>53</td>
<td>0.05274</td>
<td>119.13</td>
</tr>
<tr>
<td>70</td>
<td>0.05005</td>
<td>125.54</td>
</tr>
<tr>
<td>85</td>
<td>0.04848</td>
<td>129.60</td>
</tr>
<tr>
<td>97.5</td>
<td>0.04730</td>
<td>132.84</td>
</tr>
</tbody>
</table>

The DMPC/P85/solvent system also exists in a cubic Pn $\bar{3}m$ phase for solvent contents $\geq 53$ wt%. This phase can not be recognized at 30 wt% solvent. In stead, the $P_{\beta'}$ ripple phase obtained in the pure DMPC system at 20°C is apparently stabilized by increasing P85 weight fraction (Figure 5.8). This might be analogous to additives like cholesterol, which has shown ability to stabilize the ripple phase (Mortensen et al., 1988). Mortensen et al. also suggested that cholesterol forms complexes in the top and bottom of a zigzag structure acting as boundaries between local solid and liquid characteristics of the lipid chains. This is in agreement with our observations but conclusions of the structure do however require a more intensive study of the system, e.g. in combination with other techniques like NMR.

At very high fractions of P85, the spectra indicate that the DMPC/P85/solvent system is in a lamellar phase. This phase may be recognized as the PPO lamellar phase, which exists above $w_{P85} = 75$ wt% in the pure P85/water system (Figure 4.6).
Figure 5.8: The figure shows the SAXS spectra obtained on samples with 30 wt% solvent, at respectively (a) 20°C and (b) 35°C.

Discussion

High-temperature lamellar-phase The Pn\textsuperscript{3}m phase can not be recognized in any of the spectra obtained at 35°C. Instead a lamellar structure is observed for \(w_{P85} = 10\) wt\% and a solvent content of 53 and 70 wt\%. The repeat distance, \(d_{\text{repeat}}\), for those concentrations can furthermore be determined to 88.5 and 76.8 Å for 53 and 70 wt\% respectively. The difference in the two repeat distances can be due to a dehydration of the lamellar phase, caused by the PEO-segments of P85.

At \(w_{P85} = 10\) wt\% and a solvent content of 53 wt\%, two additional peaks can be observed at 0.11 and 0.22 Å. This leads to the suggestion that the presence of P85 copolymers cause some in-plan ordering, which is similar to the properties found in gel-phases of DMPC vesicles incorporated with cholesterol. A more plausible explanation might however be that the lamellar-phase coexist with another phase. This coexisting phase may be the same as observed for \(w_{P85} = 10\) wt\% and a solvent content of 30 wt\% at 35°C, since the phase is also found at higher P85 weight fractions.

Finally, for very high weight fractions of P85 the behavior from 20°C can again be recognized in the 35°C spectra (Figure 5.8).
5.3 Differential Scanning Calorimetry (DSC)

In order to determine the thermodynamic characteristics of the ternary DMPC/P85/solvent system a differential scanning calorimetry study has been carried out.

To overcome difficulties with overlapping peaks in the DSC spectra, the thermodynamic data are extracted by fitting the Gaussian function to each spectrum. The used Gaussian function is given by

$$C_p(T) = \frac{\Delta H}{\Delta T \sqrt{\pi/2}} \cdot e^{-\frac{(T-T_{\text{trans}})^2}{\Delta T^2}} + C_{p,0}$$

(5.2)

where $\Delta H$ is the transition enthalpy, $T_{\text{trans}}$ is the transition temperature, $\Delta T$ is the full width half max temperature, and $C_{p,0}$ is an off-set parameter.

Figure 5.9: The figure shows the specific heat capacity (arbitrary units) of the DMPC/P85/solvent system. The solvent content are kept at 70 wt% in all samples. The P85 weight fraction is given by $w_{\text{P85}}$ in units of wt%.

**Observations**

**Specific heat capacity** From Figure 5.9 it can be seen that the specific heat capacity, $C_p$, shows four major characteristics.

The most dominant feature of the system is the enthalpy-peak near $T_m \sim 24^\circ C$, where the main-phase-transition of the pure DMPC system occurs, when going from the $P_{\beta'}$ to the $L_\alpha$-phase (Figure 5.10). The main-phase-transition is due to chain
melting and order-to-disorder arrangement of the lipid molecules, separating the low-temperature $P_{\beta'}$ gel-phase from the high-temperature $L_{\alpha}$-phase.

This relative sharp transition resembles the lipid main-transition being rather sharp and continuously changing from the pure DMPC value. The ”main” peak is dominant up to $w_{P85} = 70$ wt% (corresponding to $\approx 25$ mol%), whereafter it becomes very difficult to define. From the spectra in Figure 5.9 it can furthermore be seen that the P85 weight fraction has an increasing effect on the intensity in the main-phase-transition region. It should also be noticed that the intensity of the main-phase-transition peak is roughly proportional to the amount of DMCP $(1-w_{P85}/100)$, as illustrated in Figure 5.11. It is plausible to associate this transition to chain melting in analogy with pure DMPC, thus indicating that the local lipid structure is preserved even for P85 concentrations as high as $w_{P85} = 70$ wt%.

Baekmark et al. found corresponding DSC results in DPPC vesicles incorporated with PEO-polymers (Baekmark et al., 1997). Baekmark et al. found also the width of the sharp peak is more or less constant, and consequently corresponding to the main phase transition. This is in accordance with the high order between the acyl-chains, resulting in high cooperativity in the acyl-chain melting behavior (Baekmark et al., 1997).

![Figure 5.10](image_url)

**Figure 5.10:** The figure shows the transition temperature, $T$, for the main phase (■), the pre-transition (▲), the high temperature peak transition (●) and the micelle formation transition (○) of the DMPC/P85/solvent system. The error bars indicate the size of $\Delta T/2$.

The preserved lipid bilayer structure is further supported by the second feature of the system which is an enthalpy peak near $T_p \sim 14^\circ C$, where the pre-transition of the pure DMPC system occurs, separating the $L_{\beta'}$-phase from the $P_{\beta'}$ ripple-phase (Figure 5.10). The ”pre-transition” peak is visible up to $w_{P85} = 40$ wt%, whereas it
Figure 5.11: The figure shows the transition enthalpy, $\Delta H$ (KJ/mol), for the different transitions of the DMPC/P85/solvent system. ($-\square-$) indicates the main transition, ($-\triangle-$) the pre-transition, ($-\bullet-$) the high temperature peak transition and ($-\square-$) the sum of main and high transitions. The enthalpies are normalized to the concentration of DMPC. The micelle formation transition is, opposite the other transitions, linked to the right axis and shown with ($-\bigcirc-$). The micelle formation transition is normalized to the concentration of P85.

for higher concentrations is too weak to appear. From the spectra it can furthermore be seen that the pre-transition peak shows the same development as the main-phase-transition peak both regarding temperature and enthalpy. The peak position is however more scattered. The enthalpy involved in the pre-transition (Figure 5.11) is relatively low ($\sim$1 kJ/mol) and within statistics independent of P85 content.

A third feature is found on the high temperature side of the main-phase-transition, which appears in the $C_p$ scans at P85 weight fractions $\geq$ 10 wt%. This very broad peak occurs around 26°C (Figure 5.10), and the peak’s width increases with increasing P85 weight fraction (Figure 5.9). The peak position furthermore moves towards higher temperatures when the P85 weight fraction increases from 10 to 40 wt%, whereas the peak position moves towards lower temperatures in the P85 range 40 to 70 wt%. For $w_{P85} \geq$ 70 wt%, the peak can not be resolved in our experiments. It furthermore seems that the peak evolve from the pure DMPC main-phase-transition, indicating the transition together with the "main" peak reflects some lipid chain melting characteristics.

The fourth feature also occurs as a very broad peak, spanning at a temperature range between 10 to 15°C for P85 weight fractions $\geq$ 70 wt% (Figure 5.10). The peak is also expected to occur at higher concentrations of P85, since it is assigned to the dehydration of the PPO block and the consequent formation of micelles. Similar properties are well established from pure aqueous P85 systems (Brown et al., 1991a).

The enthalpy, $\Delta H$, of the DMPC/P85/solvent system is illustrated in Figure 5.11.
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From the figure it can be seen that the enthalpy of the first, second and fourth feature are more or less constant, with only small derivations within the experimental accuracy, when the P85 weight fraction increases. The opposite happens with the third feature, where the enthalpy increases with increasing P85 weight fraction.

Discussion

In 1997 Baekmark et al. (Baekmark et al., 1997) made a study similar to ours, though performed on unilamellar vesicles of DPPC mixed with either DSPE-EO45, DSPE-Eo110 or tri42 (tri-block copolymer PEO_{42}PS_{42}PEO_{42}). Even though their results are not directly comparable with our results, due to differences in sample conditions, it is still of interest to discuss similarities and differences.

Baekmark et al. (Baekmark et al., 1997) observed a peak equivalent to the third feature in their $C_p$ scans of unilamellar DPPC vesicles mixed with DSPE-EO_{45}, at lipo-polymer concentrations between 7.5 and 30 mol%. They also showed that the main-phase-transition temperature only changes a few degree Celsius, whereas the position of the high temperature peak increases with increasing lipo-polymer concentration. The temperature difference between the "main" and high temperature peaks also increases with increasing lipo-polymer concentration and equivalent for the intensity. The behavior of the high temperature peak from Baekmark et al. is in perfect agreement with our observations.

Based on their observations, Baekmark et al. concluded that the peak on the high temperature side of the main-phase-transition cannot come from a lateral separation of DPPC and DSPE-EO_{n}, with $n = 45$ or 100, into two phases within the bilayer, since the PEO-chains start to overlap above a certain concentration of DSPE-EO_{45} or DSPE-EO_{100}. Thus, the interchain entropic repulsion forces the lipid anchors of the lipo-polymer to be placed as far away as possible from each other in the bilayer, and this hinders a lateral phase separation.

In order to describe their system Baekmark et al. suggested to use a simple 2-phase model, originally proposed by Hristova and Needham (Hristova and Needham, 1994, 1996), which takes the free energy of a system containing DSPC-DSPE-EO_{45} into account. The equation proposed for the free energy takes a very simple form and have the bilayer cohesion and polymer lateral pressure as the two main contributors. The expression is given by (Baekmark et al., 1997),

$$\frac{F}{\sigma} = \frac{F_{\text{bilayer}}}{\sigma_{\text{bilayer}}} - \frac{F_{\text{polymer}}}{\sigma_{\text{micellar}}}$$

(5.3)

where $F_{\text{bilayer}}$ is the free energy of the bilayer cohesion and $F_{\text{polymer}}$ the free energy of the lateral pressure. The $\sigma$’s are the average area per lipid molecule and the subscripts indicate that DSPC stabilizes the bilayer, whereas the lipo-polymer stabilizes the micellar structure.

At high lipid concentrations the calculation of Hristova and Needham (Hristova and Needham, 1994, 1996) leads to a predominance of the first term in Eq. (5.3), whereas the lipid/lipo-polymer arrange themselves as bilayer structures. As the concentration
increases, the lipo-polymers tend to destabilize the bilayer, whereby the micellar aggregates, consisting of both lipids and lipo-polymers, start to form in coexistence with the bilayer. According to Hristova and Needham (Hristova and Needham, 1996), the first micellar phase consists of H$_1$ micellar rods, but as the lipo-polymer concentration increases the micelles changes to spherical ones. Thus, pure DSPE-EO$_{45}$ forms spherical micelles in water (Kenworthy et al., 1995).

Based on the $C_p$ scans of lipid samples in the micellar phase, Baekmark et al. assumed the lowest lying sharp peak to be correspondent to the main-phase-transition. Thus, the width of this peak is both small and more or less constant. This is in accordance with the high order between the acyl-chains, which results in high cooperativity in the acyl-chain behavior. The width of the highest lying broad peak is on the other hand large, which is expected because of the low acyl-chain order and low cooperativity in the acyl-chain behavior. Both the lowest lying and the highest lying peaks might then reflect lipid ordering, the lowest originating from lipids in the lamellar phase and the highest from lipids incorporated in the polymeric micelles. Baekmark et al. furthermore assumed that the equilibrium between the vesicles and micelles is not affected by the temperature.

Based on the two assumptions Baekmark et al. were able to calculate the fraction, $f$, of lipo-polymer molecules in the bilayer relative to the total amount of lipo-polymers (Baekmark et al., 1997).

$$f = \frac{\Delta H_T (n_{pol}) n_{tr}}{\Delta H_T^{tr} n_{pol}}$$

(5.4)

$$\simeq \frac{\Delta H_T (n_{pol}) n_{app}}{\Delta H_T^{app} n_{pol}}$$

(5.5)

where, $n_{pol}$, is the total lipo-polymer concentration, $\Delta H_T (n_{pol})$ is the measured transition enthalpy at $n_{pol}$, $n_{tr}$ and $n_{app}$ are the threshold and the apparent threshold lipo-polymer concentration respectively, and $\Delta H_T^{tr}$ and $\Delta H_T^{app}$ are the transition enthalpy at the threshold or the apparent threshold lipo-polymer concentration respectively.

A visual comparison of Baekmark et al. and our $C_p$ scans show that the scans have similar features, especially for lower concentrations of P85. Because of the similarities the calculation of Eq. 5.5 can be applied to the obtained measurements of the DMPC/-P85/solvent system (Figure 5.12). It should be noticed that Baekmark et al. argued that micellation is strongly inhibited, due to the copolymer spanning of the bilayer, as well as the the model is developed for lipid/lipo-polymer systems consisting of bilayer and lipo-polymer rich micelles. This is obvious not in accordance with our system, but it is the best and only way to describe a system in which both high and low cooperativity between the acyl-chains can be observed.

When analyzing the results in Figure 5.12, one should be aware that the calculation is vitiated with large uncertainties. E.g. the apparent threshold concentration is assumed to be 1 mol%, which is based on results obtained by x-ray studies. The assumption $\Delta H_T^{app} = H_T (0)$ is also not true, but due to lack of measured values of $\Delta H_T^{app}$ this is our most qualified guess.

From our DSC studies it can be seen that low cooperativity between acyl-chains do appear in other phases than the micellar one. Actually, most complex between P85
Figure 5.12: The figure shows a plot of the calculated weight fraction of P85 molecules in the bilayer as function of the total concentration.

and lipid molecules will cause reduced cooperativity between the acyl-chain also when present in bilayer systems. Therefore the presence of lipids with low cooperativity between the acyl-chains do accordingly not prove the existence of micelles, but is only evidence of some degree of DMPC/P85 complex formation, e.g. as micelles, domains in the Pn̅₃n phase or small dissolved complexes.

Moreover, if the enthalpy peaks are only related to the lipid organization, one should expect the integrated enthalpy, observed as the sum of the two peaks to be constant. In the DMPC/P85/solvent system, this sum clearly increases with amount of P85, indicating tight lipid-polymer complex formation.

In our system we have no independent indications of macroscopic phase separation into a system of dominating pure lipid/water and a system of dominating P85/water (or P85/lipid/water), and there is no structural indication of polymer micelles for P85 concentrations up to \( w_{P85} \approx 50\text{wt}\% \). On contrary the x-ray data, strongly indicates mixing of polymers and lipids within one structural ordering. We therefore find it more likely that the broad peak reflects melting and disordering of lipids in near vicinity of P85 polymers, or perhaps even complexed with these, thus giving rise to significant reduced mutual cooperativity between these lipid molecules.

In the discussion of the DPPC/tri42 mixture, Baekmark et al. concluded that tri42 is incorporated into the bilayer. In comparison, and as already mentioned, P85 interacts with the bilayer either by spanning the bilayer or by entering and leaving the bilayer on the same side. Baekmark et al. furthermore considered how tri42 is stretched to span the bilayer, and to determine the hydrophobic thickness of the bilayer they used
the following expression (Sperotto and Mouritsen, 1988)

\[ \bar{d} = \frac{1}{2} \left( d_{gL}^f + d_{fL}^f \right) \approx 2.19 (m - 1) \]  

(5.6)

where \( d_{gL}^f \) is the thickness of the gel phase and \( d_{fL}^f \) the thickness of the fluid phase of the bilayer. \( m \) is the number of carbon atoms in the lipid acyl-chain. The thickness of DPPC and DMPC are determined to 32.85 Å and 28.47 Å respectively.

Assuming the maximum monomer length is 4 Å; Baekmark et al. determined the Flory radius of the polystyrene block to \( \sim 38 \) Å, which is similar to the membrane thickness. Based on this determination and the transition temperature results, Baekmark et al. suggested the PS block to be located between the two lipid layers. A cylinder conformation of the PS block, located in the space between and parallel to the acyl-chains, tends to induce disorder in the acyl-chains, which leads to significant decreases in \( T_m \). A PS block located in the space between the two layers might cause less disorder, due to the less contact between the acyl-chains and PS blocks.

The above assumptions and considerations can also be applied to our DMPC/P85/solvent system. Thus, the Gyration radius substitutes the Flory radius in the determination of the PPO-block size.

The Gyration radius is given by \( R_g = \sqrt{Nbl/6} \), where \( N \) is the number of monomers, \( l \) the length of the monomers and \( b \) the Kuhn length. Assuming \( l = 2 \) Å and \( b = 10 \) Å for PEO, and that PPO blocks have the same characteristics as PEO (Mortensen, 1996), \( R_g \) can be determined to 12 Å. This gives a diameter of 24 Å, which is less than the hydrophobic thickness of the bilayer. The monomer length \( l = 2 \) Å is however given for an aqueous solvent, but since the PPO block is in an apolar environment, PPO is not expected to form a helical structure a maximum length of \( l = 3.24 \) Å is therefore a better suggestion. By doing so \( R_g \) can be calculated to 14.7 Å, whereby the diameter equals the hydrophobic thickness. Rather than calculating the radius based on a random coil, however, one may compare the typical size of the hydrophobic PPO core of P85-micelles with that of the membrane thickness. Depending on temperature, the PPO core radius of 50 Å is typically found, i.e. the PPO chain is still much less stretched expelling the bilayer membrane, when compared to the conformation inside a micelle.

Based on the calculation it can be concluded that, P85 does not incorporate with a spherical or cylindrical shape of the PPO block through the bilayer, since no significant reduction in the transition temperature, \( T_m \), can be observed for the DMPC/P85/solvent system.
5.4 Comparison with homopolymer (PEG)

In the attempt to verify whether the P85 hydrophobic parts of the P85 block copolymers is truly incorporated into the lipid bilayer system, we have extended the study to include PEG (poly ethylene-glycol) 1000/4000 in some of our experiments. A replacement ratio of \( n_{P85} = 2n_\nu \), where \( \nu \) denotes either PEG1000 or PEG4000, has been used.

![Figure 5.13: The figure shows SAXS spectra for DMPC mixed with either P85 (■), or PEG1000 (○). For comparison pure DMPC (—) has been added to the graph.](image)

From Figure 5.13 it can be seen that the presence of 1 mol% P85 (corresponding to 6.4 wt%) results in a dramatic change in the scattered intensity of the DMPC system, whereas 1.98 mol% PEG1000 (corresponding to 1 mol% P85) only slightly alters the scattering intensity. The small increase in the Bragg peak position upon adding PEG can be explained by the dehydration of the bilayer caused by the presence of PEG1000 (Lehtonen and Kinnunen, 1995). The same development can be observed for PEG4000.

The positions of the observed Bragg-peaks for the ternary DMPC/P85/solvent and DMPC/PEG/water systems are plotted in Figure 5.14, and from the figure it can be seen that a tremendous change occurs in the peak positions and number of P85, when going from 0.5 to 1.0 mol% P85 at 20 and 35°C. The change reflects the phase transition from a lamellar phase to a cubic Pn3m phase at 20°C, and a phase transition from \( L_\alpha \) to another lamellar phase at 35°C. The presence of PEG homopolymers in the system only have minor gradual effect. At 35°C, the peak positions still undergo large changes, but the pattern of the peak positions are not similar to the ones observed at 20°C. As discussed in previous sections the DMPC/P85 system is suggested to keep the bilayer structure, while the morphology of the bilayer changes, which is due to the presences of PEO-chains. Again, no similar observations are found in the DMPC/PEG system. The lack of significant changes in the DMPC/PEG system indicates that the presences of PEG only slightly alters the lamellar morphology.

The lattice parameter of the DMPC/PEG systems decreases with increasing concentration of PEG, which is due to dehydration of the bilayer (Lehtonen and Kinnunen,
Figure 5.14: The figure shows the peak positions for spectra obtained on DMPC samples mixed with either P85, PEG1000 or PEG4000. The concentration of PEG1000 and PEG4000 are the double of P85, but equals the concentration of PEO chains in the P85 samples.

Table 5.6: The table shows the mean values of the lattice parameters for different DMPC mixtures at 20 and 35 °C. For DMPC mixed with P85 the parameters are the repeat distance, $d_{001}$, for P85 concentrations $\leq 0.5$ mol%, and the unit cell axis, $d_{100}$, for P85 concentrations $\geq 1$ mol%. For both PEG types the lattice parameter is $d_{001}$.

1995) (Table 5.6). Due to the bilayer-ordering effect of PEG, it is therefore possible to observe higher order ripple peaks for the DMPC/PEG systems in the P$_\beta$ phase.

Based on the comparative study it can therefore be concluded that the observed effects in the DMPC/P85/solvent system are caused by incorporation of PPO segments in the DMPC aggregates and not only the presences of PEO (or PEG) in the solution surrounding the bilayers.
5.5 Temperature scan of DMPC/P85/solvent system

A detailed study of the temperature dependent structure has been carried out on the DMPC/P85/solvent system by using small-angle x-ray scattering. In the experiments a DMPC/P85 mixture with \( w_{P85} = 10 \text{wt\%} \) (corresponding to 1.6 mol\%) dissolved to a solvent content of 70 wt\% has been used. The temperature scan itself has been performed by increasing the temperature from 10 to 35°C, in steps of one degree. After equilibration for 5 min, the samples are exposed for one hour. The acquired SAXS spectra are shown in Figure 5.15.

![SAXS spectra](image)

Figure 5.15: The figure shows the SAXS spectra for temperature scans of DMPC mixed with P85 at \( w_{P85} = 10 \text{ wt\%} \). A phase transition occurs around 23°C.

5.5.1 Low-Temperature Cubic Phase

From Figure 5.15 and 5.16 it can be seen that the scattering data is dominated by a large number of closely spaced Bragg peaks. The peaks start resolving at \( q \sim 0.08 \text{ Å}^{-1} \) and vanishes at \( \sim 0.16 \text{ Å}^{-1} \) for temperatures below 15°C, which is where the pre-transition peak from DSC can be observed.
From the SAXS spectra it can furthermore be seen that peaks between 0.15 and 0.23 Å\(^{-1}\) quite significantly change their relative intensities between 10 to 23°C, which may be caused by changes in the form and structure factors. The four observed peaks in the 0.15-0.23 Å\(^{-1}\) range move slightly their positions towards higher \(q\)-values with increasing temperature. Only exception is the peak positioned around 0.21-0.215 Å\(^{-1}\), which exhibits a more complex behavior.

![Figure 5.16](image)

**Figure 5.16:** The figure shows a plot of the peak positions for the DMPC/P85/solvent system. The positions are determined by fitting Lorentzian peaks to the scattered intensities, corrected for the powder averaging by multiplying \(q^2\). At 24 and 25 °C, the peak above 0.2 Å\(^{-1}\) is not present which is due to uncertainties in the determination of the position.

The low-temperature phase of the DMPC/P85/solvent system can however not be described as a simple coexistence of lamellar ordered DMPC and cubic ordered P85-micellar in terms of the published results of respectively DMPC (Janiak et al., 1979) and aqueous P85 (Mortensen, 1996) systems. This proves the low-temperature phase is a new phase where both lipids and copolymers are components.

The large number of peaks observed in the low-temperature scattering spectra is in agreement with a one-phase system of cubic space group Pn\(\bar{3}\)m. All observed peaks are reasonably well associated with this phase, though some allowed peaks in the Pn\(\bar{3}\)m structure factor can not be observed. The absence of peaks is most likely caused by a form factor minimum near 0.16 Å\(^{-1}\) from the basic structure of the lipid bilayer.

The behavior of the above mentioned Bragg peaks might be related to the enthalpy pre-transition peak observed at the same regime temperature. If this is true it should probably not be counted in the cubic structure determination. Though, the absence of this peak at low temperatures could also just be a matter of intensity in the temperature dependent product of form and structure factors.

From Figure 5.16 it can also be seen that lattice parameter decreases by \(\sim 10\) Å in the temperature range 10-23°C, going from 132 to 123 Å.

In relation to the broad enthalpy-peak observed in DSC close to 20°C, no major changes can be observed in the temperature scan. Only exception is the third order
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Table 5.7: Observed position of Bragg-peaks associated with Pn\overline{3}m cubic symmetry at 16°C

<table>
<thead>
<tr>
<th>Pn\overline{3}m</th>
<th>110</th>
<th>111</th>
<th>200</th>
<th>211</th>
<th>220</th>
<th>221</th>
<th>310</th>
<th>311</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed peak no.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>q-value (Å⁻¹)</td>
<td>0.0813</td>
<td>0.0896</td>
<td>0.10318</td>
<td>0.12341</td>
<td>0.14708</td>
<td>-</td>
<td>-</td>
<td>0.17475</td>
</tr>
<tr>
<td>Pn\overline{3}m</td>
<td>222</td>
<td>321</td>
<td>400</td>
<td>322</td>
<td>411</td>
<td>331</td>
<td>420</td>
<td>422</td>
</tr>
<tr>
<td>Observed peak no.</td>
<td>-</td>
<td>7</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>q-value (Å⁻¹)</td>
<td>-</td>
<td>0.18875</td>
<td>0.20617</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2298</td>
<td>0.2561</td>
</tr>
</tbody>
</table>

peak, which seems to vanish exactly at this temperature. Our temperature resolutions is, though, too course to verify such relationship between structure and measured enthalpy.

When the temperature increases from 22 to 24 °C the samples go through one more phase transition, changing from the cubic Pn\overline{3}m phase to a lamellar phase. This phase transition is dedicated to the melting of the acyl-chain and the disorder state of the lipids (fluid state). From the illustration of the peak positions in Figure 5.16 it is obvious that the DMPC/P85 lamellar phase is different from the pure DMPC lamellar L\text{α}-phase. This can be explained by the concentration of P85 in the bilayer, which is so high that the PEO chains overlap themselves, forcing the bilayers apart from one another. The L\text{α}-phase is dominated by equally spaced Bragg peaks as required for the lamellae structure and in our measurements identified as the (0,0,1), (0,0,2) and (0,0,3) peaks (Fig 5.16).

5.5.2 High-Temperature Lamellar Phase

From Figure 5.17 it can furthermore be seen that the repeat distance, d_{001} (lamellar), increases nearly linear with increasing temperature, going from 81 Å at 24°C to 101 Å at 35°C. This is properly caused by the PEO-chain which slightly loses its hydrophilic properties whereby the bilayers are repelled. The development may also be caused by the entropy effect, which increases the volume of the PEO chains with increasing temperature. The periodicity is significantly different from pure DMPC in the L\text{α}-phase, which has a periodicity equal to d = 65 at 35°C. The difference in periodicity is close to two times the gyration radius of the P85 unimers, R_g = 17Å (Mortensen and Pedersen, 1993), which might suggest P85 is expelled from the inner parts of the lamellae and in agreement with polymer layers on both sides of the lipid bilayer membrane in pure DMPC systems. It is also possible, though, that P85 has a large softening effect on the bilayer bending modulus, resulting in an enhanced Helfrich repulsion and consequently a larger equilibrium interlamellar distance. The anomalous swelling from pure DMPC systems can not be recognized in our DMPC/P85/solvent system.
5.5. TEMPERATURE SCAN OF DMPC/P85/SOLVENT SYSTEM

Figure 5.17: The figure shows a plot of the unit cell parameter/repeat distance vs. the temperature of the DMPC/P85/solvent system. ◦ is the unit cell axis, \( d_{100} \), of the Pn\(\bar{3}\)m phase up to 23°C, while ● is the repeat distance \( d_{001} \) for the \( L_{lamellar} \) phase above 23°C.

Despite the high temperature phase seems quite equivalent to the \( L_{\alpha} \)-phase of the pure DMPC system, we can observe an additional but relatively weak peak beyond the lamellae peaks, namely at \( q = 0.12 \) Å\(^{-1}\). The origin of this peak is still not resolved, but the peak can clearly be observed already at \( T = 24°C \) and it is therefore doubtful that this structural property is related to the 26°C-transition observed in the DSC-measurements. Opposite the three strong Bragg peaks, the \( q \)-value of this extra peak is rather independent of the temperature. Beyond 30°C the \( q \)-position merges with the third order lamellar peak and is no longer possible to observe. These distinct different temperature dependencies clearly shows that the \( q = 0.12\)Å\(^{-1}\) peak is not directly related to the lamellar thickness and periodicity. This could be due to in-plane orders within the lamellae or correlation between the P85 chains. Alternatively, the system is not a true single phase system with excess water present, at high temperatures, but has a minority component which is different from the DMPC/P85 dominating lamellae. It is however clear, that the high temperature structural properties of the system is dominated by a lamellar phase which resembles the fluid lamellar phase of pure DMPC systems, but with a significant renormalized periodicity and perhaps some weak in-plane correlations.

5.5.3 Intermediate-Temperature

The DMPC/P85/solvent system shows characteristics of both the cubic and lamellar phases at the intermediate temperature \( T = 23°C \), which is the boundary between the lamellar phase and the cubic phase. The cubic Pn\(\bar{3}\)m spectrum is however still dominating due to the large number of peaks (Figure 5.15).

A lamellar characteristic for the \( T = 23°C \) spectra is a drastic increase in the intensity of the 0.085Å\(^{-1}\) peak, which in the cubic phase equals the (1,1,1) Bragg
peak. The intensity approaches the (1,00,)-lamellar Bragg peak, which indicates a simple epitaxy relationship between the two corresponding crystal planes. Another characteristic is the broad peak ranging from 0.15 to 0.24Å⁻¹, which shape undergoes large changes when moving towards lower q-values as the temperature increases beyond the phase-transition.

The presence of lamellar characteristics indicate a coexistence of the cubic Pn\(\bar{3}\)m and lamellar phases in a small temperature range around 23°C in the DMPC/P85/-solvent system.
5.6 Phase diagram of the DMPC/P85/solvent system

Based on the results from the different experimental observations it is possible to draw phase diagrams of the ternary system at 20 and 35°C respectively.

At T = 20°C, the ternary system is in a bilayer ripple phase for low copolymer concentrations and all water contents. As the copolymer concentration increases beyond \( w_{P85} = 2 \) wt% the cubic \( Pn\bar{3}m \) phase starts to form for water content between 30 - 53 wt% and above. For water content below this concentration the system exists in a phase similar to the bilayer ripple phase, which might be analogous to other additives as e.g. cholesterol (Mortensen et al., 1988). A further increase in the copolymer concentration brings the ternary system into a regime of coexisting phases, while it at very high copolymer concentrations form phases recognized from the pristine P85 copolymer phase diagram.

At T = 35°C, the \( L\alpha \)-phase of the pure DMPC/water system is recognized at low copolymer concentrations. As \( w_{P85} \) increases for water content larger than 30 wt% the ternary system undergoes a phase transition to another lamellar phase. For water content below 70 wt% the new lamellar phase only exists in a narrow \( w_{P85} \) interval, if existing at all, and the ternary system will in this regime exist in a non-defined phase. For very high copolymer concentrations the ternary system begins to form phases recognized from pristine P85.
Figure 5.18: Phase diagrams of the DMPC/P85/solvent ternary system, with water content shown versus $w_{P85}$. 
5.7 General Conclusion on the DMPC/P85/solvent system

The SAXS measurements show that ternary systems containing 30 wt% solvent have a different behavior than systems with a higher solvent content. The differences can be seen at both 20°C and 35°C and various P85 weight fractions. At 20°C, the change in behavior seems to be caused by the stabilization of the ripples in the $P_{\beta'}$-phase; a final confirmation requires however further studies, while the change at 35°C is due to low hydration of the bilayer.

Based on differential scanning calorimetry study it can furthermore be concluded that both the pre- and the main-transitions are preserved in DMPC samples with $w_{P85} = 10$ wt% or more and dissolved to a solvent content of 70 wt%. The pre-transition peak remains visible until at least $w_{P85} = 40$ wt%, which indicates the bilayer structure is present in the system. The dominant main phase transition peak around 24°C remains in the system until at least $w_{P85} = 70$ wt%, showing a minor but systematic decrease in the temperature as the P85 content increases.

The main peak in the $C_p$ scan seems to split up into one with high cooperativity, determined as the main-transition, and a rather broad transition of low cooperativity at slightly higher temperatures. Due to the remaining presence of a sharp enthalpy peak it can be concluded that there is a high cooperativity between the acyl-chains. This behavior is only exhibited by ordered lipid molecules, which is why we conclude lipid molecules to a large extent will arrange themselves with a high degree of mutual order between the acyl-chains in the low-temperature cubic $Pn3m$ phase whereby the basic bilayer structure is preserved. This microscopic structure is perhaps similar to the one proposed by Funari et al., namely a $Pn\bar{3}m$ phase where the lipids are arranged in rods with a radius equal to half of the bilayer thickness (Funari et al., 1997).

The additional broad enthalpy peak emerging at temperatures slightly above the main-phase-transition is designated to chain melting of lipid molecules with low cooperativity between the acyl-chains. Baekmark et al. (Baekmark et al., 1997) have argued against micellisation of tri-block copolymer/lipid systems when tri-block copolymers span the bilayer. They have also observed that samples with higher copolymer concentrations cannot be prepared by extruding. Bare the results from our experiments in mind, an explanation for the problems with extruding the samples can be the existence of a non-lamellar phase, e.g. the $Pn3m$ phase. Because of the tremendous phase transition in the DMPC/P85/solvent system, at $T = 23^\circ C$, a formation of the coexisting micellar phase should not be inhibited. Therefore the Baekmark et al. argumentation is not plausible for our system. Thus, lipid molecules showing low cooperativity between the acyl-chains do not prove the existence of micelles, but is only an evidence of complex formation between P85 and the lipid molecules, indicating a kind of phase separation on the molecular scale. Such micro-scale phase separations have previously been proposed in DMPC bilayer systems incorporated with cholesterol (Mortensen et al., 1988). If micelles are present in the system then small-angle scattering in the $q$-range below 0.1 Å$^{-1}$ should be expected. No such scattering is however observed in the SAXS spectra obtained on the DMPC/P85/solvent system,
which is why the appearance of micelles may be rejected as a plausible structure. The DMPC/P85 complex existing as domains in the Pn\(\bar{3}m\) phase may also be rejected, since the phase transition occurs above the main-phase-transition, leading to a change in the structure from Pn\(\bar{3}m\) to lamellar. Based on this, it is not possible to suggest a better explanation than a complex between P85 and DMPC exists in the system.

It can furthermore be concluded that the DMPC/P85/solvent system is in agreement with the Pn\(\bar{3}m\) cubic symmetry up to \(\sim 23^\circ\text{C}\), for samples with \(w_{P85} = 10\text{ wt}\%\) and dissolved to a solvent content of 70 wt\%. Above the main-phase-transition temperature, the system is in a lamellar phase and the repeat distance, \(d_{001}\), for this lamellar phase is larger than the corresponding lamellar \(L_\alpha\)-phase for the pure aqueous DMPC/solvent system. The lamellar phase of the DMPC/P85/solvent system shows further a marked increase in \(d_{001}\) with increasing temperature, going from 78 Å at 24\(^\circ\text{C}\) to 97 Å at 35\(^\circ\text{C}\), which might be caused by the PEO segments or the entropic effect. Thus, PEO segments become less hydrophilic with increasing temperature which forces the bilayers apart and leads to an increasing PEO-chain volume due to the entropic effect. In comparison, the pure aqueous DMPC/solvent system has a repeat distance of app. 65 Å. Other phase transitions can also be observed in the SAXS spectra, but due to lack of time they have not been closely studied.

From the temperature scan of the high-temperature phase, an additional small Bragg peak has systematically been observed. The origin of this peak is still unknown to us, but one may speculate in some in-plane order within the lamellae similar to the properties found in gel-phases of DMPC vesicles incorporated with cholesterol. A more plausible explanations is however some kind of coexistence between the lamellar-phase and another phase.

Based on the comparison with homopolymer it can also be concluded that incorporating P85 into DMPC/solvent systems causes marked changes already at relatively low P85 concentrations. The changes can not only addressed to the dehydration of the system, since incorporating PEG only have relatively minor effects on the basic lamellar structure. P85 is therefore conclusively incorporated into the bilayer.

Finally, from a medical point of view the obtained structural and thermal characterization of the DMPC/P85/solvent system shows that when using liposomes as drug carrier only small concentrations of P85 can be used in order to obtain steric stabilization. Thus, even at low P85 fraction the system will undergo phase transitions to other structures than vesicles. Determination of P85’s ability of steric stabilizing liposomes require how ever further studies.
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Reference


