4 Interaction of polyarginine with PG containing membranes

4.1 Introduction

Despite the large interest in the so called “arginine magic” (see chapter 2.2) only few articles are available dealing with well defined model systems to elucidate the detailed binding mechanism of arginine polypeptides to lipid membranes.

Tsogas et al. (2005a; 2006; 2005b) examined the interaction of arginine monomers, polyarginine and guanidinylated dendrimers with di-hexadecyl-phosphate (DHP) containing vesicles. They reported that arginine monomers influence the lipid phase behaviour, as the main transition temperature $T_m$ is decreased and the transition enthalpy $\Delta H$ is increased. ITC experiments showed that arginine monomers binds stronger to gel phase DHP-membranes than to fluid DHP-membranes. The authors further argued that some monomers penetrate the membrane and are transported into the vesicles' interior (Tsogas et al. 2005b). From $\zeta$-potential and fluorescence experiments they concluded that polyarginine is located at the vesicles interface as long as the membrane is in the gel state, but that it penetrates the membrane in the liquid crystalline state (Tsogas et al. 2005a). Guanidylated dendrimers induced fusion of the vesicles and a reduction of $T_m$. Under certain conditions the dendrimers might also be translocated into the vesicles interior (Tsogas et al. 2006).

In contrast to these findings Goncalves et al. (2005) reported that nonaarginine (R₉) neither inserts nor translocates through a POPG/POPC (3:7) bilayer, but binds within some distance to the headgroup region. They determined high binding constants ($8.2 \times 10^4$ M⁻¹) and binding enthalpies (-2.5 kcal/mol) from ITC experiments and evaluated the electrostatic contribution of binding to be 33 % of the total free energy.

Similar calculations, yielding similar values, were performed by Hitz et al. (2006) on the basis of fluorescence experiments. They reported that PLA binding to POPG/POPC (7:3) membranes leads to bilayer rigifications followed by the release of the aqueous content of the vesicle. Furthermore, they showed that PLA binding to the vesicles follows a two step kinetic, with a first step being probably electrostatically driven and a second one non-electrostatically.

This work shall contribute to the understanding of the binding mechanism of PLA to negatively charged membranes. It is not the objective of the work to examine the translocation through the lipid bilayer. Rather, we want to reveal the mechanism of binding, which always has to precede a possible translocation. A focus point is the influence the peptide binding has on membrane properties. Our model system comprises PLA of different chain length and PG vesicles and monolayers. The influence of PLA binding on the membranes phase behaviour
was determined in a series of DSC experiments. ITC studies revealed the binding characteristics in both, the gel and the fluid membrane state. Mechanistic information was deduced from monolayer experiments. FT-IR spectroscopic experiments revealed the influence of PLA binding on the membrane organization and hydration as well as the secondary structure of the peptide. Finally, dye release experiments were performed to examine the possibility of pore formation in the vesicles, induced by polypeptide binding.

These different experiments were used to deconvolute electrostatic and non-electrostatic contributions to the binding process. The existence of these two contributions will be evident from all the presented data.

After insight into the PLA binding process is achieved, it will be compared to PLL binding, which was discussed in the foregoing chapter.

### 4.2 Differential scanning calorimetry

#### 4.2.1 Influence of PLA on the phase behaviour of DPPG membranes

As in the case of PLL interaction a series of DSC experiments was performed under variation of i) the peptides chain length ii) the lipid-to peptide mixing ratio $R_c$ and iii) the membrane composition.

DSC curves of pure DPPG and its complexes with PLA of different chain lengths at a mixing ratio of $R_c = 1$ are presented in Figure 4.1. The PLA chain length was varied in 4 steps from 69 to 1183 monomer units. The phase transition temperature of pure DPPG (40.8 °C) and the transition enthalpy (10.7 kcal/mol) agree well with the values reported in literature (Durvasula and Huang 1999; Huang and Li 1999; Zhang et al. 1997). By addition of PLA this transition is only marginally affected. Nevertheless, a small effect is seen. The two shorter peptides, PLA 69 and PLA 184 decrease $T_m$ slightly, by $\Delta T_m = -0.8$ °C and $-0.2$ °C, respectively. The two longer PLAs ($n = 649$ and $n = 1183$) increase $T_m$ by as little as $\Delta T_m = 0.2$ °C and 0.1 °C, respectively. These shifts are unexpectedly small. In general one would expect a much higher positive shift upon binding of a positively charged polyelectrolyte to the negatively charged DPPG bilayer. Shielding the electrostatic repulsion between neighbouring lipid molecules reduces their lateral distance and results in an increased hydrophobic contact of the acyl chains. This and the connected gain in Van-der-Waals energy stabilizes the gel phase and shifts the transition to higher temperatures. The shift that can be attributed to such electrostatic shielding of the PG headgroup charges ($\Delta T_{el}$) amounts to 5.5 °C (Cevc et al. 1980). This behaviour was detected for PLL binding to DPPG membranes (Schwieger and Blume 2007). All effects that exceed $\Delta T_{el}$ or produce a negative $\Delta T$ originate from - or have a contribution of - non-electrostatic interactions, such as specific binding
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including hydrogen bridging), structural changes, changes in hydration, or hydrophobic interactions. This is definitely the case for PLA binding to DPPG bilayers. Certainly, PLA binding leads to electrostatic screening of the headgroup charges. But this effect must be counteracted by opposing effects that shift the phase transition to lower temperatures. These different effects are balanced in a way that the overall shift of $T_{m}$ is close to zero. This thesis is supported by the fact that positive as well as negative shifts are observed for PLA of different chain length. It can be assumed, that the principle effects of binding are the same for all PLA, but that these effects are balanced differently for PLA of different chain length, resulting in a slightly positive $\Delta T_{m}$ in the one case and in a slightly negative $\Delta T_{m}$ in the other. In general, chain length dependencies are expected for polyelectrolytes binding to charged surfaces. This arises from the fact that the main driving force of binding is the entropy win achieved by counterion release (Montich et al. 1993; Wagner et al. 2000). This effect is greater the more counterions are released upon the binding of one polymer chain. Consequently, the binding constant, which has an entropic and an enthalpic contribution, is getting larger for longer polyelectrolyte chains. This increase in binding constant is counteracted by sterical and kinetical effects (Novellani et al. 2000; Ulrich et al. 2006). Thus generally trends reverse at a certain chain length, as has been shown for the DPPG/PLL system (see chapter 3 or Schwieger and Blume 2007).

**Figure 4.1:** DSC-plots of the gel-to-liquid-crystalline phase transition of DPPG/PLA complexes with an equimolar charge ratio ($R_{c} = 1$) and different PLA chain length. The vertical dotted line is added as an eye guide at the phase transition temperature of the uncomplexed lipid membrane. Measurements are performed in 100 mM NaCl solution at pH = 6.
The pre-transition that is observed for the uncomplexed DPPG bilayer at 34.6 °C is abolished upon addition of PLA. The pre-transition is the transition from the gel phase Lβ temperature / °C to the ripple phase Pβ'. It is very sensitive to changes in the bilayer environment and structure. A suppression of the pre-transition was reported for different peptides and anaesthetics (Heimburg 2000) as well as for high salt concentrations (Blume and Garidel 1999) or Small Unilamellar Vesicles (SUV). In the present case screening of the surface charges, changes in hydration structure (see IR section), and a pronounced stabilisation of the lamellar bilayer geometry might be responsible for the disappearance of this transition.

The enthalpy of the main transitions (ΔH$_{\text{main}}$) is affected as well and shifted to slightly lower values. ΔΔH$_{\text{main}}$ is about -1.5 kcal/mol for all PLA/DPPG complexes with respect to the main transition enthalpy of the pure DPPG membrane. With respect to the overall gel to fluid transitional enthalpy, which includes the pre-transition enthalpy (ΔH$_{\text{gel->fluid}}$ = ΔH$_{\text{pre}}$ + ΔH$_{\text{main}}$), the decrease is even higher and results in values of about -2.5 kcal/mol. Since the transition enthalpy is given by

$$\Delta H_{\text{trans}} = T \cdot \Delta S_{\text{trans}}$$

a decrease in the transition entropy is expected if the transition temperature stays more or less constant and the transition enthalpy decreases. A reduced ΔS$_{\text{trans}}$ indicates that the fluidization of the membrane is hindered by PLA binding.

Finally attention shall be focused on changes in half width of the transition peaks. The half width is inversely correlated with the cooperativity of the melting process. The cooperative unit (c.u.), being a measure for the number of molecules that change their phase state at the same time (Garidel et al. 2000b), can be calculated on basis of a simple two state model (Gel ⇄ fluid) (Trauble 1971), yielding:

$$c.u. = \frac{\Delta H_{\text{vH}}}{\Delta H_{\text{calor}}} = \frac{4RT^2C_{\text{max}}}{\Delta H_{\text{calor}}^2}$$

with ΔH$_{\text{vH}}$ being the van ‘t Hoff Enthalpy of melting, evaluated from the temperature dependence of the equilibrium constant and ΔH$_{\text{calor}}$ being the integral of the Δc$_p$ curve.

From Figure 4.1 it is obvious, that the cooperativity is decreased upon binding of the shortest PLA and increased upon binding of the longer ones. In the latter case the c.u. is increased from 108 to about 180 molecules. An increased cooperativity is however, an astonishing result. There are many examples for decreased cooperativities upon peptide binding, whereas no example for an increased cooperativity of the lipid main transition is reported. An increased cooperativity must be due to a better lateral coupling of the lipids in the bilayer, which might be facilitated if the headgroup charges are screened by the binding peptide. Hitz et al. (2006) report that fluid POPC/POPG bilayers are rigified upon PLA interaction. It might be possible that the cooperativity in such rigified domains is increased, resulting in a sharper transition peak. Interestingly Tsogas et al. (2005b) reported that the sub-
transition of a DHP membrane showed an increased cooperativity after addition of arginine monomers. Nevertheless, also in their experiments the main transition is broadened, i.e. the cooperativity is decreased. They further report a reduction of $T_{m}$ upon interaction of DHP containing bilayers with arginine monomers. This supports our thesis, that there must be an arginine specific process that is destabilizing the gel phase of the membranes. Nevertheless they find, contrary to our results, an increase in $\Delta f_{\text{trans}}$.

![DSC plots](image)

**Figure 4.2:** DSC plots of the gel-to-liquid crystalline phase transition of DPPG vesicles complexed with a) PLA 69 and b) PLL 1181 as a function of the lipid/Arg mixing ratio ($R_c$). The vertical dotted line is added as an eye guide at the phase transition temperature of the uncomplexed lipid membrane. Measurements are performed in 100 mM NaCl solution at pH = 6.

In Figure 4.2 the DSC curves of DPPG/PLA complexes at different mixing ratios $R_c$ are presented. $R_c$ is always given with respect to the charges under the assumption, that the DPPG molecules as well as the arginine side chains each carry one negative and one positive charge, respectively. This assumption is reasonable as the experiments were performed at a pH of 6 - 7, which is far from the two $pK_{\text{app}}$ values which are 2.9 and 12.5 for DPPG and PLA, respectively. This means that the DPPG is completely deprotonated and PLA is completely protonated.

Obviously two situations can be distinguished, which are the lipid excess and peptide excess complexes. The transition temperatures $T_m$ of the peptide excess complexes ($R_c < 1$) are always decreased with respect to $T_m$ of a uncomplexed DPPG membrane. The $\Delta c_p$ curves change neither position nor size and shape below a $R_c$ of 0.5, indicating that excess peptide doesn’t influence the membrane organisation any more but remains unbound in the solution. Nevertheless, a change is still visible between the curves at $R_c = 1$ (which is charge compensating ratio) and $R_c = 0.5$. This allows us to conclude that complexes formed under peptide excess are overcharged, leading to a surface charge reversal. This is a common effect
for polyelectrolyte binding (May et al. 2000; Netz and Joanny 1999) and is used extensively to
build polyelectrolyte capsules by layer-by-layer deposition (Decher 1997; Sukhorukov et al.
1998). The extent of overcharging depends on the PLA chain length, as can be deduced from
the fact that the curves measured at \( R_c = 1 \) are more resemblant to the lipid excess behaviour
for longer PLA chains and to the peptide excess behaviour for shorter PLA chains. This means
that the saturation limit shifts to lower \( R_c \) values, when the PLA chain gets longer, i.e. the
overcharging is more pronounced for longer PLA chains. This can be easily understood,
because unbound loops and ends of the polymer that extend in the solution are responsible for
the overcharging effect and these loops and ends are getting more frequent with increasing
polymer chain length (Chodanowski and Stoll 2001). In the lipid excess regime (\( R_c > 1 \) \( T_m \))
is unaffected (longer PLA) or decreases slightly (shorter PLA). It is obvious that here the \( \Delta_{cp} \)
peaks change continuously in position, shape, peak heights and integral area dependent on the
peptide content. Peaks get smaller, i.e. transitions get more cooperative with increasing peptide
content, with the highest cooperativity being achieved at the saturation ratio. This can be
explained by all of the lipid molecules being in one and the same, namely the bound, state.

The transition enthalpy \( \Delta H^{trans} \) increases with the increasing peptide content but is always
lower than the transition enthalpy of the unbound membrane (\( \Delta H^0 \)). Papahadjopoulos (1975)
describes two typical situations. Binding of charged peptides to membrane surfaces leads to a
\( \Delta H^{trans} \) that is higher than \( \Delta H^0 \) and increases with increasing peptide content. Conversely,
insertion of the peptide into the membrane leads to a \( \Delta H^{trans} \) that is lower than \( \Delta H^0 \) and
decreases with increasing peptide content. The measured dependency for PLA binding to PG
membranes does fit in none of these models. The fact that \( \Delta H^{trans} \) is always lower than \( \Delta H^0 \)
suggest that PLA inserts into the membrane. However, the increase of \( \Delta H^{trans} \) with increasing
peptide content, suggest an electrostatic interaction. This ambiguous behaviour leads once
more to the conclusion that PLA binding cannot be explained by one of these simple one step
models, but is rather an interplay of different processes.

Also the peak symmetry is different in lipid and peptide excess complexes. Whereas the
lipid excess complexes show shoulders and extended wings on the high temperature side of the
transition peaks, the peptide excess transitions extend more to the low temperature side. An
asymmetric peak is indicative for a better interaction of the added peptide with one of the two
phases. If the peak shows an extended wing or shoulders at the low temperature side, the
peptide interacts better with the fluid phase than with the gel phase, if the peak extends to the
high temperature side, the interaction with the gel state is favoured. The wings arise from an
accumulation of the peptide in one or the other phase in the two phase region of the melting
process (Ivanova and Heimburg 2001). Following this interpretation, we suggest that in the
overcharged complexes PLA binding to the fluid phase is favoured, whereas at lower peptide
content PLA binding to the gel state of the membrane is favoured.
4.2.2 Influence of PLA binding on the miscibility of DMPC/DPPG membranes

By adding the zwitterionic DMPC to the membrane the surface charge is reduced, which has effects on binding constants, saturation concentrations and steric constraints during the binding process. Moreover the two kinds of lipids may phase segregate and form differently composed domains. Such a demixing has been shown for PLL binding to mixed membranes (see chapter 3.2.2). It is believed that demixing plays an important role in biological systems to provide specific environments for protein binding and/or activation. Since PC and PG lipids show nearly identical bilayer structure (Watts et al. 1981), transition temperatures and enthalpies (Durvasula et al. 1999), these two molecules are ideally miscible if they have the same acyl chain lengths (Garidel et al. 1997). The difference of two CH₂ units in the acyl chain length of DMPG and DPPG leads to negative nonideality parameters, which point to a tendency for complex formation (Garidel et al. 1999). Nonetheless the coexistence range is small and the DSC curves of the mixtures show one cooperative phase transition. Despite this small nonideality, we chose this system as a means to monitor lipid demixing, because demixing results in two distinguished transition peaks.

![Figure 4.3: DSC plots of the binary lipid mixture DMPC/DPPG complexed with various amounts of PLA 184 resulting in the indicated charge ratios (RC) of lipid charge / peptide charge. The vertical dotted lines indicate the main phase transition temperature of pure DMPC, the uncomplexed lipid mixture DMPC/DPPG = 1/1 and pure DPPG(from left to right). Measurements are performed in 100 mM NaCl solution at pH = 6.](image-url)
The influence of PLA addition on the phase transition of an equimolar mixed DMPC/DPPG membrane is shown in Figure 4.3. It is obvious that the transition peak splits in at least two components upon PLA addition. The two components are shifted to lower and higher temperatures, respectively. The higher component, which is centred at around 36 °C, is increasing its intensity with increasing peptide content, whereas its position is rather stable. The lower component decreases in intensity and is shifted to lower temperature, as the peptide content increases. This behaviour can be interpreted with partial demixing of the lipids. PLA induces the formation of a PG rich domain, with a transition shifted to higher temperature. PLA is bound to this domain. The rest of the lipids are gathered in a consequently DMPC rich uncomplexed domain, with a lower transition temperature. That PLA does only interact with the negatively charged DPPG and not with the neutral DMPC was tested in a control experiment (data not shown). The more peptide is added, the more lipids get organised in the domain with bound PLA, depleting more and more DPPG in the other domain. Therefore, the transition temperature of the DMPC rich domain continuously decreases. In contrast, the DPPG rich binding domain seems to be unchanged in composition, which is probably favourable for PLA binding.

![Figure 4.4](image_url)

**Figure 4.4:** Binary lipid mixture of DMPC and DPPG in an 1/1 mixing ratio (mol/mol) (red) and complexed with PLA of different chain lengths in amounts to give a final charge ratio \( R_c = \text{DPPG/PLA monomer} \) of 1. The vertical dotted lines indicate the main phase transition temperature of pure DMPC, the uncomplexed lipid mixture DMPC/DPPG = 1/1 and pure DPPG (from left to right). Measurements are performed in 100 mM NaCl solution at pH = 6.

However, in the transition curve of the overcharged complex \( R_c = 0.5 \) a separated DMPC component is not well resolved and the \( \Delta c_p \) maximum is shifted downward with respect to the
complex of $R_c = 1$. This is in agreement with Denisov et al. (1998), who state that domains dissipate if the total polyelectrolyte charge exceeds the total lipid charge.

The data presented in Figure 4.3 are data from the second heating scan. Data from the second cooling scan show in principle the same behaviour, but the component at lower temperature shows a more pronounced supercooling than the one at higher temperature. This supports the explanation that the two peaks originate from separate processes (e.g. the melting of separate domains) and not from a very asymmetric transition.

A further indication for this interpretation arises from the characteristic changes the transition peaks undergo in dependency of the PLA chain length. The corresponding DSC curves are presented in Figure 4.4. The shortest PLA ($n = 69$) affects the transition only marginally, whereas all longer PLAs induce splitting of the transition peak into three components. The high temperature component, which is assigned to the PG rich peptide bound domain, increases in intensity and cooperativity with increasing PLA chain length. The low temperature component, reflecting the transition of PC enriched membrane parts, is considerably broader, indicating a less well-defined structure of these membrane parts.

A third component can be identified which does not change position with respect to the transition midpoint of the uncomplexed membrane. This indicates that a certain fraction of lipids is not accessible for the binding polymer. This might be the inner lamella of multilamellar vesicles or precipitated vesicles that are not in equilibrium with the supernatant, or sterically or electrostatically shielded surfaces. The described trend of changes in transition behaviour is interpreted with a higher domain formation capacity of the longer polypeptides. This is in good agreement with models presented in literature (Franzin and Macdonald 2001) and was also observed for PLL induced domains. According to Macdonald et al. (1998) domain areas are proportional to the square root of the polyelectrolyte mass. Nevertheless, there seems to be an upper limit for this relationship. This is concluded from the fact that the $\Delta c_p$ curves of membranes complexed with the two longest PLAs ($n = 649$ and $n = 1183$) basically resemble.

The finding of domain formation led us to investigate more thoroughly the effect of PLA binding on the miscibility of mixed DMPC/DPPG membranes. According to Hac et al. (2005) domain formation is not necessarily reflected by macroscopic phase separation. If the domains are too small they are not recognized as independent phases. Therefore, the question arises whether the domain formation results in a miscibility gap in the phase diagram. Hence, we performed more experiments with different DMPC/DPPG mixing ratios. The DSC curves of different mixed membranes without and with PLA in a charge-to-charge mixing ratio of $R_c = 1$ are presented in Figure 4.5a.

The transition curves for the complexes containing PC excess over PG are considerably broadened, gaining intensity at the high temperature side. In addition all the peaks show the above-mentioned structuring in two or three components. The complexes containing more PG than PC show increased phase transition cooperativity upon complexation with PLA. This was
already shown to be a property of pure DPPG/PLA complexes, which consequently turns up again in the DPPG rich complexes.

**Figure 4.5:** a: DSC plots of the phase transition range of different binary lipid mixtures DMPC/DPPG (−) and of the respective mixtures complexed with adequate amounts of PLA 184 to yield a lipid-to-peptide charge ratio of one ($R_c = 1$) (−). b: possible phase diagram for the mixture DMPC/DPPG being complexed with PLA 184 constructed from $T_{on}$ (●) and $T_{off}$ (■) of the red graphs shown in a.

On- and offset temperatures of the transition curves ($T_{on}$ and $T_{off}$) were used to construct a phase diagram for DMPC/DPPG mixtures with bound PLA (Figure 4.5b). The liquidus curve, which is given by the mole fraction dependence of $T_{off}$, increases continuously. Thus there is no indication for lipid demixing in the liquid crystalline phase. However, the liquidus curve is asymmetric showing a steep increase at low PG mole fractions. $T_{on}$, which determines the solidus curve, is nearly constant for the membranes with low PG content, including the 1/1 PC/PG mixture. This is an indication for a gel phase miscibility gap. Mixtures containing more PG than PC show a continuous increase in $T_{on}$. Thus for those membranes no demixing is expected.

However, $T_{on}$ and $T_{off}$ can not be determined very precisely and depend strongly on the baseline that is defined during the data analysis. It is especially not clear whether the solidus curve at low PG contents is horizontal or not. The plateau in the solidus curve is not as nicely pronounced as in the case of PLL binding. It might also be that the solidus curve shows a slight increase in this region of the phase diagram. Moreover using $T_{on}$ and $T_{off}$ as delimiter of the phase boundaries is only a very rough estimation, because peak broadening by low cooperativity is not taken into account (Johann et al. 1996). To validate the hypothesis of a gel
phase miscibility gap more proof is necessary. Another attempt to investigate the miscibility will be described in the IR section (section 4.5).

4.3 Isothermal titration calorimetry

After DSC experiments indicated that at least two processes are involved in PLA binding to DPPG membranes that influence the lipid phase transition in a different manner, we performed more experiments to elucidate the character of these processes. Isothermal titration calorimetry (ITC) is capable of monitoring minute changes in binding enthalpy upon stepwise addition of one reactant to the other. Compared to the PLL experiments, the titration was performed in the reversed manner (titrating PLA into PG vesicle suspension). In the case of PLA binding, this gave the better experimental results.

Figure 4.6 shows the titration curves and the integral heats of the reaction of different PLA with DPPG vesicles at 10 °C below $T_m$. The heat profiles clearly show that the binding of one adequate PLA to DPPG involves two processes, one being endothermic and the other being exothermic. Up to a binding of approximately 0.4 equivalents of PLA ($R_c = 2.5$) the binding is endothermic. Further titration to higher peptide contents leads to an exothermic reaction, which is indicating a change in binding mechanism. The highest amount of heat is released at a peptide content of 0.5 ($R_c = 2$). Further titration results in a decay of the reaction enthalpy to zero. Fitting these decays with a simple one site binding model yields binding constants in the range of $10^3$ to $10^4$ M$^{-1}$.

The same experiments performed at a temperature 10 °C above $T_m$ (see Figure 4.7) show that the binding of PLA to fluid membranes is exothermic for all titration steps. Nevertheless, the initial decrease of the reaction enthalpies might be due to an endothermic process that is still underlayed but over-compensated by the exothermic one. But also a cooperative binding would explain the initial decrease. Cooperativity could be due to structural changes either of the membrane or the peptide during the binding process that facilitates subsequent binding. It is possible that a change of the peptides’ secondary structure upon binding produces such an effect. Yet, also the reduction of dimensionality during the binding process of polyelectrolytes produces an apparent cooperativity (Mosior and McLaughlin 1992b).

The maximal heat is released at a binding ratio of about one. Exceeding this ratio results in a steep decrease of the reaction enthalpy per titration step, indicating high binding constants ($10^5$ to $10^7$ M$^{-1}$) and a 1 to 1 binding stoichiometry.
**Figure 4.6:** Titration curves of gel phase DPPG vesicles (LUV, \( r = 50 \) nm, 2 mM total lipid) with PLA of different chain length with (20 mM) at 30 °C. **Top:** heating power vs. time. **Bottom:** integral heats of reaction per mole of injectant (PLA) vs. mixing ratio.

**Figure 4.7:** Titration curves of fluid phase DPPG vesicles (LUV, \( r = 50 \) nm, 2 mM total lipid) with PLA of different chain length with (20 mM) at 50 °C. **Top:** heating power vs. time. **Bottom:** integral heats of reaction vs. mixing ratio.
The binding of PLA to fluid state vesicles is clearly chain length dependent, which is more clearly seen, if the total heat of reaction per mol of lipid is plotted against the molar ratio (see Figure 4.8). To calculate these heats, the heats per injection have to be corrected by a volume factor that reflects the displacement of cell content during each injection. Heats are detected only in the cell volume and not in the supernatant solution. For the calculation it is assumed that supernatant and cell content always have the same composition and that the reaction takes place after the injection volume has been displaced from the cell. According to these considerations the following equation was derived to calculate the total heat of reaction per mol of cell content (in this case: lipid):

\[
Q_{\text{cell}}^{\text{tot}} = \sum_{i} \left[ \frac{Q_{i}^{\text{inj}} \left( 1 + \left( \sum_{j} v_{i}^{\text{inj}} / v_{\text{cell}} \right) \right)}{c_{0}^{\text{cell}} \cdot v_{\text{cell}}} \right]
\]

(12)

with \( i \) being the number of injection, \( Q_{i}^{\text{inj}} \) and \( v_{i}^{\text{inj}} \) the heat and the volume of injection, \( v_{\text{cell}} \) the cell volume and \( c_{0}^{\text{cell}} \) the concentration of the reactant in the cell before the first injection.

With increasing PLA chain length the total heats of reactions as well as the binding constants increase, as can be concluded from their shape of the titration curves. Comparison of the binding of PLA of the same chain length to gel state and fluid vesicles shows that binding to fluid state vesicles is much more exothermic than to gel state vesicles. The differences in reaction enthalpies range between -0.8 kcal/mol (PLA 69) and -1.5 kcal/mol (PLA 649). As binding to the two states of the membrane and the phase transitions of pure and bound membrane can be regarded as a cyclic process these heats are connected according to:

\[
\Delta H_{L/P}^{g \rightarrow f} = \Delta_f H_L^{g \rightarrow f} + \Delta_B H_f^{g} - \Delta_B H_f^{g}
\]

(13)

with \( \Delta H_{L/P}^{g \rightarrow f} \) being the gel to fluid transition enthalpy of the lipid/peptide complex, \( \Delta_f H_L^{g \rightarrow f} \) the transition enthalpy of the free lipid, and \( \Delta_B H_f^{g} \) and \( \Delta_B H_f^{g} \) the binding enthalpies of the peptide to the fluid and the gel phase, respectively. Thus the binding enthalpies should contribute to the phase transition enthalpy measured in the DSC experiments (see Figure 4.1 and Figure 4.2). If, as in the here presented case, the difference between the last two terms in equation (13) is negative (i.e. exothermic), the transition enthalpy of the membrane/PLA complex is reduced with respect to the transition of the free membrane. This explains, at least partly, the unexpected reduction of transition enthalpies which were reported above.
The ITC experiments give direct evidence for at least two distinct binding processes, but still mechanistic information is lacking. Nevertheless, besides the different binding constants and enthalpies it was observed that also the binding stoichiometry of PLA binding to gel phase or fluid phase membranes is different. Whereas binding to fluid vesicles shows a 1 to 1 stoichiometry, gel state vesicles seem to be saturated at a peptide content of 0.5 \( R_c = 2 \) or even below. A stoichiometry of two lipids per peptide monomer is expected, if one assumes, that binding is electrostatic and that only the lipids of the outer monolayer of the vesicle are accessible. This explains the gel state binding stoichiometry. A stoichiometry of 1, as found for PLA binding to fluid vesicles, would consequently mean that all the lipids of a vesicle are accessible, i.e. the vesicle is ruptured or the peptide is translocated through the membrane.

Indeed, arginine rich macromolecules have been found in the lumen of vesicles and cells after application to the outside (Mitchell et al. 2000; Rothbard et al. 2004; Sakai et al. 2006; Wender et al. 2000). The pathway of translocation is an issue of an ongoing discussion and not yet clear (see introduction).

We showed by cryo-TEM imaging (Figure 4.25), that fluid POPG vesicles are not completely ruptured upon PLA binding. Moreover we performed dye release experiments that showed that the vesicle content does not completely mix with the external volume (see 4.6). Thus, it might be that transient pores (Tang et al. 2007) are formed that allow the peptide to migrate into the vesicle and to bind to the inner monolayer.
The notion that more PLA interacts with membranes in the fluid state than in the gel state, was also reported by Tsogas et al. (2005a), who showed by zeta potential measurements that more PLA was necessary to neutralise anionic DHP containing vesicles in the fluid phase as compared to the gel phase. Moreover they showed by fluorescence quenching that the PLA content in the bulk phase was reduced to a higher extent in the presence of fluid vesicles than in the presence of gel phase vesicles. They argued that PLA is partially incorporated in the lipid bilayer at temperatures above $T_{\text{m}}$.

Translocation pathways through the hydrophobic core of the membrane are discussed in the published literature (Fuchs and Raines 2006). Indeed it was shown that PLA might be transferred into and transferred through hydrophobic environments (e.g. chloroform, octane) after complexation to amphiphilic anions (Rothbard et al. 2004; Sakai et al. 2006). Also anionic phospholipids have been shown to alter the hydrophilicity of PLA drastically facilitating a transport to hydrophobic media (Sakai and Matile 2003; Tang et al. 2007). Hitz et al. (2006) state that only 25–30 % of the free energy of interaction between PLA and POPG containing vesicles is of electrostatic origin. The rest is attributed to hydrogen bonding and/or hydrophobic interactions.

Temperature dependent ITC experiments showed that hydrophobic interactions indeed play a role in PLA/PG complex formation (see Figure 4.9). It can be seen that the enthalpies of the titration of PLA 649 into a suspension of fluid POPG vesicles decreases with increasing temperature. The temperature dependence of $\Delta R_H$ gives according to equation (8) the change in heat capacity during the reaction. A negative $\Delta R_C^p$ is indicative for a release of water from
hydrophobic surfaces (Blume 1983; Garidel and Blume 1999), thus implying that hydrophobic interaction between PLA and the membrane take place or the hydrophobic contact between the membrane lipids is intensified. $\Delta \rho C_p$ is also influenced by ion binding to the membrane surface with subsequent release of hydration water from the interaction zone. However, these effects would produce a positive $\Delta \rho C_p$ and thus counterbalance the effect that arises from hydrophobic dehydration. It was shown that the electrostatic binding of oligoarginine (R$_9$) to heparinsulfate (a polyanion) produces a positive $\Delta \rho C_p$ (Goncalves et al. 2005). Thus it is unlikely that the negative value we measured can be attributed to any aspect of headgroup interaction. $\Delta \rho C_p$ of PLA binding to POPG vesicles was estimated to be -17.7 cal mol$^{-1}$K$^{-1}$ (-74 J mol$^{-1}$K$^{-1}$), which formally corresponds to the removal of two hydrocarbon hydrogen atoms from contact with water (Gill and Wadso 1976). But since the hydrophilic contribution to $\Delta \rho C_p$ is not known this value remains uncertain. The same temperature dependence could also be shown using saturated DPPG instead of POPG (data not shown).

### 4.4 Monolayer experiments

To further elucidate the mechanism of PLA binding to PG membranes, we performed monolayer experiments at the air water interface. A monolayer is a very simplified membrane model, mimicking only one leaflet of a membrane. Thus, not all processes that take place in volume phases can be observed. Possible processes such as aggregation, fusion, pore formation, or translocation that might account for energetic effects, cannot be examined in monolayer experiments. But the reduction in possible responses to PLA binding is also a strength of these experiments. Only the first steps of binding are detected and can be separated from subsequent structural changes that often make volume experiments difficult to interpret.

Monolayers of DPPG were spread on an aqueous subphase containing 100 mM NaCl at different surface pressures $\pi_0$. After injection of PLA into the subphase we recorded the changes of surface pressure as a function of time (Figure 4.10). The shape of the recorded surface pressure curves is strongly dependent on the initial pressure $\pi_0$. Two general situations can be distinguished: Is PLA injected underneath a monolayer at low $\pi_0$, the surface pressure decreases first and increases in a second step, reaching an equilibrium value higher than $\pi_0$. If PLA is injected underneath a monolayer at high $\pi_0$, the surface pressure increases, reaching a plateau ca. 20 min after injection.

Which of the two cases is observed seems to be dependent on the phase state of the monolayer. Monolayers can exist in different phases. At low surface pressures ($\pi$) and high areas per molecule ($A_m$) the monolayer is in the liquid expanded phase (LE), which is, with respect to lipid order and mobility (but not in the headgroup cross sectional area), comparable to the liquid crystalline phase ($L_{\alpha}$) of a bilayer. At higher surface pressure and low area per
molecule the monolayer is in the so called liquid condensed phase (LC) which is comparable to the gel phase ($L_{\beta}$) formed by bilayers. The transition pressure ($\pi_t$) observed under the here chosen conditions is 10 mN/m (see Figure 4.11 or underlayed $\pi-A$ isotherm in Figure 4.10).

**Figure 4.10:** *Left:* Adsorption kinetics of PLA 184 at DPPG monolayers at different starting surface pressures on a subphase of 100 mM NaCl solution in H$_2$O. PLA (10 µl, 15 mM) was injected underneath the monolayer at $t = 0$. The red curve (top scale) is the surface pressure/Area isotherm of DPPG at 20 °C, which is given to identify the monolayer phase state. **Right:** Changes in surface pressure after injection of PLA vs. initial surface pressure.

The decrease in surface pressure upon PLA binding to LE monolayers indicates that the lipids get condensed and the molecular area and mobility is lowered. This is due to electrostatic adsorption to the interface and shielding of the headgroup charges. The subsequent increase in surface pressure is interpreted as an insertion of the arginine side chains into the headgroup region of the monolayer, thus, decreasing the molecular area per lipid molecule. This binding mechanism is also found in the LE/LC phase transition region.

When all lipids are in the condensed state ($\pi_0 > 15$ mN/m) no decrease of $\pi$ is detected upon PLA interaction but the injection is now directly followed by an increase of $\pi$. This increase is higher the higher the initial surface pressure of the monolayer is. The lack of the initial decrease implies that lipids, which are organised in a liquid condensed monolayer are not further condensed by PLA adsorption. The peptide side chains, though, still insert between the lipid headgroups. At the monolayer-bilayer equivalence pressure, which was found to be ca. 30 mM/m (Blume 1979), PLA addition increases the surface pressure, i.e. the peptide inserts into the monolayer. Because at this pressure the lipid organisation in the monolayer
should be the same as in a bilayer at the same temperature, it can be assumed that also gel state
DPPG vesicles are penetrated by PLA.

The differences of pressures reached after 1 h of interaction and the initial values \( \pi_0 \) are
given in Figure 4.10b. Interestingly these \( \Delta \pi \) values are almost constant in the range of
\( \pi_0 < 20 \text{ mN/m} \), which is the range of LE monolayers and the coexistence range LE/LC. When
all lipids are in the condensed state \( \Delta \pi \) increases with \( \pi_0 \). This relationship is quite unexpected,
because commonly reported relationships are inverse, i.e. \( \Delta \pi \) depends inversely proportional
on \( \pi_0 \), which is explainable with the peptide being more readily inserted in a more loosely
packed monolayer (Bringezu et al. 2007; Demel et al. 1973; Dyck and Loesche 2006;
Kimelberg and Papahadjopoulos 1971; Maget-Dana 1999).

An explanation for the increasing \( \Delta \pi \) values with increasing \( \pi_0 \) can be given under
inspection of the \( \pi-A \) isotherms, which are presented in Figure 4.11. The isotherm of DPPG on
a pure NaCl subphase shows a transition pressure \( \pi_t \) of 10 mN/m, with a corresponding
molecular area of 80 Å² and a collapse area of 40 Å². These values correspond well with
DPPG isotherms, reported in the literature (Grigoriev et al. 1999; Sacre and Tocanne 1977). If
DPPG is spread on PLA containing subphases the molecular area per lipid is increased in the
LE phase as well as in the LC phase, indicating an insertion of PLA into the monolayer. This
confirms the interpretation of the pressure increase detected in the adsorption experiments. The
\( \Delta \pi \) values given above correspond to the pressure difference of the pure DPPG isotherm and
the isotherm of DPPG on a PLA containing subphase at a constant molecular area (see up
pointing arrows in Figure 4.11). This pressure difference increases when the compressibility of
both monolayers decreases under compression (i.e. the isotherms become steeper). The effect
of an increasing pressure differences is even more pronounced when the compressibility of the
DPPG monolayer with inserted PLA decreases more than that of the pure DPPG monolayer.
This can be, indeed, observed for the isotherms of LC phase monolayers (see Figure 4.11).
This increasing difference in surface pressure between both isotherms with decreasing \( A_m \) is
equivalent to the increase in \( \Delta \pi \) with increasing initial monolayer pressure \( \pi_0 \), which was
observed in the adsorption experiments.

This correlation exists as long as the inserted polypeptide is not squeezed out from the
monolayer at the exclusion pressure \( \pi_{ex} \). In the case of PLA inserted into DPPG monolayers \( \pi_{ex} \)
is higher than 40 mN/m. The high exclusion pressures indicate a strong interaction between the
lipids and inserted PLA. The high exclusion pressures are also responsible for the unusual
relation between \( \Delta \pi \) and \( \pi_0 \).

The lower compressibility of monolayers with bound PLA as compared to pure DPPG
monolayers indicates that the insertion of PLA leads to a better order of the lipids. Probably,
the monolayer compensates for the area reduction per lipid molecule, that is caused by PLA
insertion, by ordering of lipid molecules that are still in the unordered LE phase.
angle microscopy showed that such unordered LE domains still exist at surfaces pressures significantly higher than $\pi_r$ (Vollhardt et al. 2000).

![Figure 4.11: Surface pressure vs. Molecular area isotherms of DPPG, spread on different subphases: ▐100 mM NaCl, ▐100 mM NaCl + 0.5 mM PLA 69, ▐100 mM NaCl + 0.5 mM PLA 184, ▐100 mM NaCl + 0.5 mM PLA 1183. The dotted lines indicate the transition pressure of the respective monolayers. The upward pointing vertical arrows indicate the expected pressure increase in the adsorption experiments, performed at constant area. The downward pointing arrows indicate the decrease of $\pi_r$ upon polypeptide adsorption.]

Also the reduction of the transition pressure $\pi_r$ shows that the interaction with PLA favours the formation of the LC phase. The reduction of $\pi_r$ is similar to the increase in the transition temperature of DPPG bilayers (see DSC section). Figure 4.11 shows that the extent of $\pi_r$ decrease depends on the PLA chain length. The longer the PLA chain, the lower is $\pi_r$. This correlates well with the increase in $T_m$ with increasing PLA chain length that was detected by DSC.

The $\pi$-A isotherms further show that also the magnitude of increase in average molecular area per lipid molecule dependents on the PLA chain length. In general, the increase is higher the longer the PLA chain is, which implies that longer PLA insert to a higher extent into the monolayer than shorter ones.

The monolayer experiments support the interpretation of the DSC data as well as of the ITC data given before. The increase of $\pi$ after injection of PLA and the increased molecular areas in the $\pi$-A isotherms show that the peptide interacts not only superficially but inserts to a certain extent in the lipid membrane or monolayer respectively. This differs from the interpretations of Goncalves et al. (2005). They stated on basis of $^2$H-NMR experiments that the interaction of R$_9$ with POPG/POPC bilayers is only superficial. However, these differences might be due to the different degrees of polymerisation.
In principle, the same effects have been found for PLL interaction with DPPG monolayers. However, direct comparison of the $\Delta \pi$ values measured in adsorption experiments shows that the pressure decreases (LE phase) are more pronounced in the case of PLL adsorption and the pressure increases (LC phase) are more pronounced in the case of PLA adsorption. This shows that PLL has a higher propensity to condense the monolayer and PLA has a higher propensity to insert into the monolayer. This is in good agreement with the results of the other experiments presented before.

4.5 Infrared spectroscopy

Transmission FT-IR spectroscopy was used to separately monitor the influence of PLA binding on different parts of the bilayer. We evaluated the changes of the methylene and carbonyl stretching vibrational bands to retrieve information on the hydrophobic and interfacial region of the membrane, respectively. In addition we monitored the secondary structure changes the peptide undergoes upon binding to the membrane by analysing the amide I absorption band. Furthermore, the influence of PLA binding on the lateral phase separation in mixed DMPC/DPPG membranes was examined. All IR experiments were performed with a lipid to peptide mixing ratio of $R_c = 1$ and as a function of temperature.

4.5.1 Complexes with pure DPPG

The CH$_2$ stretching bands

The frequency of the CH$_2$ stretching vibration ($\nu$(CH$_2$)) is sensitive to the order of the hydrocarbon chains in the hydrophobic part of the membrane. In Figure 4.12 the wavenumbers of the $\nu_s$(CH$_2$) vibrational bands of DPPG and its complexes with PLA of different chain length are presented as a function of temperature. The transition temperature $T_m$ of the membrane can be determined from the point of inflection. It is only marginally influenced by PLA binding. In the case of PLA 184 binding it is slightly decreased ($\Delta T_m = -0.9 ^\circ C$) and in the case of binding PLA 69 and 649 it is slightly increased ($\Delta T_m = 0.4 ^\circ C$). The absolute values are below the resolution of the experiment and should not be over-interpreted. However, they reproduce very well the results of the DSC experiments.

Additional information can be deduced from the absolute values of the vibrational frequencies. In both, the gel and the liquid crystalline phase the wavenumber of $\nu_s$(CH$_2$) is reduced upon interaction of the membrane with PLA. This effect was already seen for the interaction with PLL, which in contrast to PLA, significantly increased the transition temperature of the membrane. This shows that the two effects are independent.
Figure 4.12: Wavenumber of the maximum of the $\nu_2(\text{CH}_2)$ vibrational band of DPPG ($\bigtriangledown$) and DPPG in the complexes DPPG with PLA 69 (■), PLA 184 (●), and PLA 649 (▲). The Lipid-to-peptide mixing ratio $R_c = 1$. Measurements are performed in 100 mM NaCl solution in D$_2$O, at pD = 7.

A lower CH$_2$ stretching vibrational frequency is normally interpreted with a higher order of the acyl chains (Tamm and Tatulian 1997). One major contribution could be attributed to the ratio of gauche to trans conformers within one acyl chain (Cameron et al. 1981). This interpretation has been confirmed by the analysis of the CH$_2$ wagging bands which are very sensitive to the number and sequence of gauche and trans conformers within the hydrocarbon chain (Senak et al. 1991; Tuchtenhagen et al. 1994).

But also other effects influence the band positions. Large influence has the nature and the charge of the lipid headgroup. Protonation (and thus neutralization) of the PG headgroup results in a reduction of the CH$_2$ stretching vibrational frequencies (Tuchtenhagen et al. 1994). The same is the case for PA headgroups. This can be attributed to a reduction in electrostatic repulsion between adjacent headgroups, which allows a closer contact between the acyl chains and increases the van der Waals interaction energies. Stronger interchain interaction is thus reducing the $\nu$(CH$_2$) frequencies. The same explanation can be adopted for the electrostatic screening by PLA binding (as it was already done for PLL binding).

However, in the present case this explanation remains unsatisfactory in one aspect. In the case of PG or PA protonation, as well as in the case of PLL binding, the increased hydrophobic contact results in an increase of $T_m$. This is not the case for PLA binding. Thus, there must be still another contribution. It was found that the stretching vibrational frequencies are influenced by other factors than acyl chain ordering. Kodati et al. (1994) showed for DPPC and hexadecane that partial deuteration of the hydrocarbon chains increases the frequencies of $\nu_2$(CH$_2$) and $\nu_{ad}$(CH$_2$), whereas the order parameters are not influenced. Equally, Kerth (2003) showed that the CH$_2$ stretching vibrational frequencies of DPPC membranes are increased if
some of the lipids had perdeuterated chains or one of the lipids acyl chains (sn-1 or sn-2) was deuterated. This effect was explained by a reduction of interchain vibrational coupling. Vice versa, this means that a decrease of the stretching vibrational frequencies, as it is observed in the present case, could be due to an increased interchain vibrational coupling. This could be accomplished by a restriction of the rotational motion of the acyl chains, induced by peptide headgroup interaction. If that is the case, an additional effect should arise from the coupling of the librotorsional modes with the methylene stretching mode (Kodati et al. 1994). If the librotorsional motion is hindered by polypeptide binding, the reduction of the $\nu_s(CH_2)$ frequency should be even enhanced.

![Figure 4.13](image)

Figure 4.13: Wavenumber of the maximum of the $\nu_s(CH_2)$ vibrational band of a) DPPG (▲) its complex with PLA 649 (▼) and b) DPPG in a mixed DPPG/d$_{62}$-DPPG membrane (□) and its complex with PLA 649 (■).

To prove the influence of interchain vibrational coupling we examined the complexes of PLA formed with mixed DPPG/DPPG-d$_{62}$ membranes, which are chemically and structurally identical to a DPPG membranes (Figure 4.13). As a result of the isotopic dilution, the interchain vibrational coupling of the CH$_2$ groups is disturbed. This is reflected by the higher wavenumber of the $\nu_s(CH_2)$ vibration of the mixed membrane if compared to a completely undeuterated membrane ($\Delta \tilde{\nu} = 0.32 \text{ cm}^{-1}$). When the peptide now interacts with the isotopically diluted membrane, the interchain coupling cannot be increased (even if the geometry is more favourable), because the CH$_2$ chain cannot couple with the neighbouring CD$_2$ chain. As a result, the $\nu_s(CH_2)$ frequency in the DPPG/d$_{62}$-DPPG/PLA complex is decreased to a much lesser extent ($\Delta \tilde{\nu} = -0.3 \text{ cm}^{-1}$) than in the DPPG/PLA complex ($\Delta \tilde{\nu} = -1.2 \text{ cm}^{-1}$). This result proves that the frequency decrease is not interpretable solely on the basis of a higher hydrocarbon order. Rather, an important contribution arises from
increased interchain vibrational coupling due to peptide headgroup interaction. The ν(CH₂) frequency decrease that is still observed in the isotopically diluted sample is explainable with the fact, that the isotopic dilution is only statistical and the coupling of the two adjacent acyl chains of one lipid molecule is not hindered in any case. Furthermore, reduced intrachain coupling of librational modes with the CH₂ stretching vibrations might still account for some νs(CH₂) decrease, because this effect, being an intramolecular one, is not affected by isotopic dilution.

With this new finding we also reinvestigated the polylysine complexes of DPPG (Figure 4.14). In principle, the same effects are visible in this system. The decrease of the νs(CH₂) frequency is about the same in the PLL and the PLA sample as long as completely undeuterated membranes are used (Figure 4.14a). If the membrane is isotopically diluted this decrease is reduced in both cases.

![Figure 4.14](image)

**Figure 4.14:** Wavenumber of the maximum of the ν(CH₂) vibrational band of a) DPPG (▽) its complex with PLL 402 (▼) and b) DPPG in a mixed DPPG/d62-DPPG membrane (□) and its complex with PLL 402 (■).

However, if the isotopically diluted membrane is complexed with PLL, the wavenumber of ν(CH₂) is still more decreased than in the case of complexation with PLA (Δν = -0.7 cm⁻¹ as compared to -0.33 cm⁻¹). This means that in the case of PLL binding indeed, part of the wavenumber reduction can be attributed to a conformational ordering of the acyl chains, as it was proposed in section 3.3. This higher influence of the ordering is also reflected by the fact that PLL increases Tₘ of a DPPG membrane, whereas PLA does not.
The lipid C=O band

As stated above, the C=O stretching vibrations of the lipid ester groups respond to the degree of interfacial hydration (Blume et al. 1988; Lewis et al. 1994). Thus, they provide a good tool to assess the degree of water penetration, which can be influenced by polypeptide binding. The broad band that is observed at about 1740 cm\(^{-1}\) is a superposition of at least two underlying components, which can be demonstrated by the second derivative spectra (Figure 4.15b). The two underlying bands arise from non hydrated carbonyl groups (ca. 1741 cm\(^{-1}\)) and a carbonyl monohydrate (ca. 1722 cm\(^{-1}\)) (Blume et al. 1988). During the phase transition, the molecular area is increased, which allows more water molecules to penetrate into the headgroup region. As a consequence, the contribution of the lower frequency component to the overall CO vibrational band increases. This is demonstrated in Figure 4.15 for a DPPG/PLA complex. It can be seen that the C=O vibrational band is broad in the liquid crystalline phase and does not have a well-defined maximum. This prevents the simple analysis that was applied in section 3.3. Hence, we performed a more detailed analysis by fitting the bands with two underlying components and comparing their intensities and positions.

![Figure 4.15](image)

**Figure 4.15:** Carbonyl stretching vibrational bands (\(\nu(CO)\)) of the complex DPPG + PLA 1183 at temperatures close to the gel to liquid phase transition region (a) and its second derivatives (b). The lipid-to-peptide mixing ratio \(R_c = 1\).

As fitting parameters for the band positions, we used the minima determined from the second derivative spectra and allowed a variance of \(\pm 1\) cm\(^{-1}\). As fitting functions, simple Gaussians were used. A combination of Gaussians and Lorenzians did not give better results. Before fitting, a baseline was subtracted in the spectral region of 1660–1800 cm\(^{-1}\). The results of the analysis of different DPPG/PLA complexes at temperatures below and above the phase transition are depicted in Figure 4.16.
In general, the band component indicating the presence of hydrated C=O groups, looses intensity as DPPG is complexed with PLA. However, the hydration of gel phase membranes is only marginally influenced by PLA binding. Binding of the shortest PLA \((n = 69)\) does not change the integral intensities of the two components, at all. The longer PLAs \((n = 184\) and \(n = 1183\)) increase the contribution of the non-hydrated C=O group by some percent. This effect seems to be chain length dependent. The longer the PLA the higher is the contribution of the non-hydrated C=O group. The lower frequency component not only looses intensity but also shifts to slightly lower wavenumber. This means that the carbonyl groups are less hydrated when PLA is bound to the membrane but the remaining water molecules are bound by stronger hydrogen bonds. This is probably due to the fact that the water molecules, being trapped between membrane and peptide, are restricted in their reorientational motion which results in better oriented hydrogen bonds (Laroche et al. 1991). This coincides also with the conclusions drawn from the \(\nu(CH_2)\) band analysis, namely that also the lipid molecules are hindered in their rotational diffusion.

In the liquid-crystalline phase, more pronounced effects are observed. By complexation of the membrane with PLA, the contribution of the non-hydrated carbonyl species is now increased by about 10%. This means that the fluid phase of the membrane is much less hydrated if PLA is bound. During the \(L_\beta \rightarrow L_\alpha\) phase transition water does not permeate into the membrane headgroup region to the same amount, because bound PLA reduces the accessibility of the carbonyl groups. Also in the fluid phase, the wavenumber of lower frequency component is shifted to lower values, indicating that hydration water forms more directed hydrogen bonds. In contrast to the gel phase complex, the fluid phase complex shows no chain length dependency with respect to hydration. The short PLA 69 has the same influence as the longer ones.

These findings complement well the results discussed above. Also the ITC experiments showed a dehydration, which could be deduced from the negative \(\Delta_R C_p\) values. Furthermore, monolayer experiments showed that LE monolayers get condensed upon PLA binding, which reduces the intermolecular space necessary for hydration. Moreover, inserted PLA side chains reduce the space available for water molecules and compete with the lipid C=O groups for the remaining water of hydration.
Figure 4.16: Carbonyl stretching vibrational bands of DPPG (top panels) and its complexes with PLA of different chain length (lower panels) at 20 °C (left panels) and 70 °C (right panels). The experimental bands (■) are fitted with 2 Gaussians (—, —), representing two distinct states of hydration. The summation of the fit components is shown in red (—). The relative integral intensities of the single components are given as percentages underneath the respective fitting curve. The vertical dotted lines indicate the positions of the fit components of pure DPPG.
The secondary structure of PLA in solution

The secondary structure of the peptide plays an essential role during the binding process and influences the thermodynamics and the structure of the formed complex, as has been already shown for the DPPG/PLL complexes. A convenient tool to determine the secondary structure of the peptide is the analysis of the amide I absorption band. Unfortunately, the data about the secondary structure of PLA available from literature are not as comprehensive as it is the case for PLL. No IR experiments have been published that can serve as a good reference system for the identification of different secondary structure elements which contribute to the amide I band contour of PLA. The use of the general band assignments that are developed for proteins (Goormaghtigh et al. 1994a, 1994b; Goormaghtigh et al. 1990; Tamm and Tatulian 1997) is questionable because the amide absorption of homopolypeptides often deviates from the general case. Therefore, we undertook a series of measurements to identify the frequencies of the amide I bands of PLA in different secondary structures.

PLA has been thoroughly studied in solutions of different chemical composition by CD spectroscopy. This gave us the opportunity to re-evaluate the same systems via FT-IR spectroscopy. The most comprehensive study was done by Ichimura et al. (1978). They examined the influence of different mono- and multivalent counterions on the secondary structure of PLA and proposed that a specific binding of tetrahedral anions leads to a coil to helix transition. Thereby, the anion should be able to bridge two guanidyl residues via electrostatic interactions and hydrogen bonding. In pure water PLA was found to form a random coil. Anions that induce an α-helix formation are SO$_4^{2-}$, ClO$_4^-$, P$_2$O$_7^{4-}$ at neutral pH as well as SO$_3^{2-}$, CO$_3^{2-}$ and HPO$_4^{2-}$ at higher pH. Increasing the ionic strength using other salts, results in a formation of β-structures and precipitation of the PLA. In context with membrane binding it is worth to mention that H$_2$PO$_4^-$ is not inducing an α-helical conformation of PLA. Miyazaki et al. (1978) reported that PLA helices formed in ClO$_4^-$ solution, unfold to give a random coil at higher temperature. The melting temperature is dependent on the ClO$_4^-$ concentration. They report as well that PLA helices are more stable than PLL helices, i.e. the free energy of helix formation is more negative. Rifkind (1969) showed that PLA helices were formed in dioxan/water mixtures. X-ray diffraction studies on the secondary structure of PLA were performed by Suwalsky and Traub (1972). They showed that the secondary structure of PLA is dependent on the water content of the sample. Hydrating PLA with 20 water molecules per arginine monomer, results in the formation of β-sheets. On reducing the water content to 5 molecules per monomer or less, α-helices are formed. Whether helix formation can be induced by pH increase, as it was shown for PLL, is controversially discussed in literature (Ichimura et al. 1978; Rifkind 1969). Clear is that the high pK$_{app}$ of PLA (12.5) makes this approach difficult (Sakai et al. 2006; Sakai and Matile 2003).
Figure 4.17: Amide I and guanidinium vibrational bands of poly(L-arginine) in random coil (100 mM NaCl, pH=7), β-sheet (100 mM NaCl, pH = 13), and α-helical (150 mM NaClO₄, pH = 7) conformation (left) and its second derivatives (right).

With this information we re-investigated the described systems via IR spectroscopy. Figure 4.17 shows amide I bands of PLA in three different secondary structures. A peculiarity that impedes the interpretation of these bands is the superposition of the amide I band with the arginy] side chain vibration. However, single components can be deconvoluted in the second derivative spectra. The symmetric and antisymmetric guanidyl stretching vibrations give rise to absorption bands at 1584 cm⁻¹ and 1608 cm⁻¹, respectively (Barth and Zscherp 2002; Chirgadze et al. 1975).

As reference system for the random coil structure, we used PLA dissolved in pure water at pH 7. In this conformation, the amide group absorbs at 1644 cm⁻¹. Spectra of PLA in 100 mM NaCl solution did not differ from those recorded in salt free solution. Thus, it is assured that in all reported experiments PLA is added in a random conformation. The α-helical conformation was induced by a 250 mM NaClO₄ solution (Ichimura et al. 1978; Martinez et al. 2007; Miyazaki et al. 1978). The amide I band of α-helical PLA is shifted to slightly higher wavenumbers (ca. 1648 cm⁻¹). The same component could be identified in dioxane and SO₄²⁻ solution, however, to a lower extent. The existence of a helix was proven by thermal unfolding, following the experiments of Miyazaki et al. (1978) (Figure 4.18). As the temperature is raised, the band at 1648 cm⁻¹ vanishes and a band at the position that was assigned to random conformers evolves. This process is completely reversible on cooling.
Figure 4.18: Amide I and guanidinium vibrational bands of PLA 649 in 100 mM NaClO₄ at different temperatures (a) and their second derivatives in the amide I region (b). Note that the order of the graphs is reversed in (b).

More difficult to indentify was the typical amide I band position of a β-sheet structure. Raising the pH of a PLA solution above the pK<sub>app</sub> shifts the band position to lower wavenumber (Figure 4.17). In the second derivative spectra, several components are visible, with the lower frequency components probably being due to β-sheet structures. To unambiguously assign a frequency to the amide I vibration of a β-sheet, we performed experiments at low water content, as suggested by Suwalsky and Traub (1972). A sample with a D₂O content of 20 molecules per side chain was prepared and measured in an open IR cell, allowing the water to evaporate in course of the measurement. To assist the evaporation, we performed heating and cooling cycles. Under these conditions a β-sheet should transform into an α-helix during the measurement. The results are presented in Figure 4.19. All detected spectra consist of two components, of which one is the typical α-helix vibrational band. The other component showing up between 1620 cm⁻¹ and 1625 cm⁻¹ is consequently assigned to a β-sheet vibration. As the temperature increases, the α-helix component decreases and the β-sheet band increases and shifts to slightly lower wavenumbers. At high temperature a small random coil component is visible. On cooling, the whole process is reversible, but due to evaporation of the water the α-helix to β-sheet transition is shifted to higher temperature (Figure 4.19). This is in good agreement with the finding that at low water content PLA α-helices are more stable than β-sheets (Suwalsky and Traub 1972) and is taken as confirmation that the proposed band assignments are reasonable.
Combining this information, we are now able to assign typical vibrational frequencies to distinct secondary structures. The results are summarized in Table 2. The high frequency component that is present in all discussed spectra is assigned to turn structures.

**Table 2**: Left: Wavenumbers of amide I vibrations of poly(L-arginine) in different secondary structures. Right: Wavenumber of side chain vibrations of Poly(L-arginine).

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Wavenumber of amide I band / cm(^{-1})</th>
<th>Side chain vibration</th>
<th>Wavenumber / cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sheet</td>
<td>1620 – 1630</td>
<td>(\nu_{as}(\text{CN}_3\text{H}_5^+))</td>
<td>1584</td>
</tr>
<tr>
<td>random coil</td>
<td>1642 – 1644</td>
<td>(\nu_{ad}(\text{CN}_3\text{H}_5^+))</td>
<td>1608</td>
</tr>
<tr>
<td>α-helix</td>
<td>1646 – 1648</td>
<td></td>
<td></td>
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<tr>
<td>turns</td>
<td>1670 - 1680</td>
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**Secondary structure of PLA bound to DPPG membranes**

With the knowledge of the band positions in the amide I region, we then examined the secondary structure PLA adopts upon binding to DPPG membranes. Figure 4.20 shows spectra in the amide I and guanidyl stretching vibration region of PLA of different chain length bound to DPPG at 10 °C and 70 °C, respectively. The band shape is as well dependent on the length of the PLA as on temperature. With increasing chain length the band is shifted to lower vibrational frequencies, indicating an increase in β-sheet elements. With increasing
temperature the band position is shifted to higher frequencies, i.e. in the region that was
assigned to random coil and $\alpha$-helical structures. The temperature dependence is most
pronounced for the PLA of intermediate chain length ($n = 184$). Interestingly also the guanidyl
stretching vibrations are influenced by membrane binding. Independent on PLA chain length
and temperature, they are shifted to lower wavenumbers. This must be due to an interaction of
the arginine side chain with the phospholipid headgroups. A downshift of the guanidyl
stretching vibrations was also shown in $SO_4^-$ solution (Chirgadze et al. 1975), which is known
to bridge adjacent arginine side chains via hydrogen bonding (Ichimura et al. 1978). Thus, the
measured downshift of the bands is an indication that PLA interacts with DPPG headgroups
via hydrogen bonding. However, we measured a downshift of the guanidyl bands also in
dioxane/D$_2$O solution and in very high concentrated saline (2 M NaCl). Therefore, also
dehydration could be responsible for the effect.

![Figure 4.20](image.png)

**Figure 4.20:** Left: Amide I and guanidyl bands of PLA of different chain length bound to DPPG
membranes at 10 °C (solid lines) and at 70 °C (dotted lines) and right: its respective second derivatives.

The second derivative spectra of the amide I region reveal that a mix of structural elements
is present in every sample. This is illustrated in detail in Figure 4.21. The spectra of the two
longer PLA ($n = 184$ and $n = 1183$) show 3 distinct components at 1645.5 cm$^{-1}$,
1639.5 cm$^{-1}$ and 1627 cm$^{-1}$. In the spectra of PLA 69 only the two higher frequency
components are present. Furthermore, in all spectra a high frequency component at 1671 cm$^{-1}$
is resolved, which is due to turn vibrations. The lowest component can be unambiguously
assigned to $\beta$-sheet structures. The two remaining components at 1639.5 cm$^{-1}$ and 1645.5 cm$^{-1}$
should arise from random coil and $\alpha$-helix vibrations, respectively. This is suggested by the
sequence of the bands in the spectra. However, the frequencies are slightly lower than those
determined for PLA in solution. This might be caused by the lower polarity of the membrane.
headgroup region as compared to bulk water. This should strengthen the intramolecular H-bonds of the peptide, which shifts the absorption maxima of the amide I vibration to lower frequencies.

Following this interpretation, we can describe the binding behaviour as follows: Longer PLA binds in a mixture of helix, random and β-sheet conformation to DPPG membranes at low temperatures. The β-sheet content increases with increasing PLA chain length. As the temperature is raised, first, the β-sheet unfolds to give more peptide in random conformation. At higher temperature, more α-helix is formed on expense of the random coil conformers. Short PLA does not form a β-sheet upon binding. The short peptides are bound mainly in a random conformation at low temperatures. As the temperature is increased, the peptide transforms increasingly into α-helices. The reason for the presence of different secondary structure motives in one sample might be the existence of differently bound peptide. Peptide that is superficially adsorbed adopts most likely another secondary structure than peptide that is inserted in the headgroup region. That both binding mechanisms exist has been shown by monolayer and ITC experiments (see above). However, the secondary structure of the bound peptide does not directly depend on the lipid phase state, as no distinct change is observed at \( T_m \).

Figure 4.21: Second derivative spectra in the amide I region of different PLA bound to DPPG membranes at \( \sim 10 \, ^\circ C, \sim 20 \, ^\circ C, \sim 30 \, ^\circ C, \sim 40 \, ^\circ C, \sim 50 \, ^\circ C, \sim 60 \, ^\circ C \) and \( \sim 70 \, ^\circ C \). The vertical dotted lines indicate vibrational components due to different structural elements.

Despite all experimental efforts, the band assignments in the spectral region between 1638 cm\(^{-1}\) and 1646 cm\(^{-1}\) remain somewhat uncertain. Alternatively to the given interpretation the higher frequency band at 1645.5 cm\(^{-1}\) could be assigned to random coil vibration. The lower frequency band at 1639.5 cm\(^{-1}\) would then arise from a conformation that was not present in bulk solution. For instance it is known that 3\(_{10}\) helices (Prestrelski et al. 1991) or bent helices (Jackson et al. 1989) have amide I bands at lower wavenumbers than α-helices.
Also different unordered structures are imaginable, for instance, a more extended conformation in the fully charged state in solution and a more coiled structure that forms upon charge compensation at the membrane surface. The assignment of the band at 1639.5 cm$^{-1}$ to an ordered structure would satisfy the expectation that all ordered secondary structure elements thermally unfold.

Which of the two interpretations is valid can not be decided at the moment. Clear is, however, that longer PLA bind in β-sheet conformation to gel phase DPPG membranes. This is different to the binding behaviour of PLL, which formed solely α-helices and random coils at the membrane surface.

4.5.2 Complexes with mixed DMPC/DPPG membranes

Miscibility of DMPC-d$_{54}$ and DMPA

To complement the DSC studies about the influence of PLA on the miscibility of PC/PG bilayers, we performed IR experiments on the same system. We used the same experiments as were described above in the PLL section (3.3.2), the only variation being the substitution of the DPPC-d$_{62}$ component by DMPC-d$_{54}$. This proved to be the better model system to study domain formation in the case of PLA binding because the transition temperatures of eventually formed domains are better separated. This approach was already used for the DSC experiments.

Figure 4.22 shows the experimental results. In pure DMPC-d$_{54}$/DPPG mixed membranes, the frequencies of CD$_2$ and CH$_2$ stretching vibrations show the same temperature dependence. This indicates that the lipids are completely miscible. The transition temperature of the mixed DMPC-d$_{54}$/DPPG membrane is 31.3 °C. After addition of PLA, the miscibility is reduced. The temperature dependence of the ν(CH$_2$) and ν(CD$_2$) is now different. The DMPC component (deuterated acyl chains) melts in a very wide transition range beginning at 24 °C and ending at the temperature where also the transition of the DPPG component is completed. In contrast, the DPPG component undergoes a ν transition in a small temperature range. The midpoint of the DPPG transition is increased with respect to $T_m$ of the uncomplexed lipid mixture. The increase is the higher, the longer the PLA chain is.

These findings are in excellent agreement with the results of the DSC experiments (see Figure 4.4). They can be interpreted with the presence of a DPPG enriched binding domain with a well defined composition and a less defined DMPC enriched reservoir. The melting begins in the membrane regions that are the most depleted in DPPG molecules somewhat higher than the transition temperature of pure DMPC-d$_{54}$ (19.7°C) (Fidorra et al. 2007). This indicates the possible presence of a gel-gel miscibility gap, at compositions of $x_{\text{DPPG}} < 0.5$. 
Figure 4.22: a: Maxima of symmetric methylene stretching vibrational bands of either component of a mixed DMPC-d$_{54}$/DPPG membrane (top panel) and its complexes with PLA 184 (middle panel) and PLA 649 (lowest panel). The CD$_2$ stretching vibrations (■), originating from the DMPC component, are shown in blue (right scale) and the CH$_2$ (▲) stretching vibrations, originating from the DPPG component, are shown in black (left scale). For comparability the left and the bottom scale is the same for all three panels. The vertical dotted lines indicate the midpoint of transition of the DPPG component (steepest slope). b: The first derivatives of the curves presented on the left hand side in the transition range. The colours are chosen as in the left panels.

The broad transition range of the DMPC component opposed to the rather sharp DPPG transition indicates a very asymmetric gel/liquid crystalline phase transition region. The PLA binding domain is DPPG enriched but is not pure DPPG. This can be deduced from the transition temperature, which is considerable below $T_m$ of a pure DPPG membrane (41 °C). Furthermore, this is indicated by the absolute values of the CH$_2$ stretching vibrational frequencies, which are not significantly decreased with respect to the $\nu_s$(CH$_2$) frequencies of the uncomplexed lipid mixture. Following the interpretation given above (section 4.5.1), this shows that the interchain coupling of the $\nu_s$(CH$_2$) vibrations is still reduced by intercalated CD$_2$ chains, which, belong to admixed DMPC-d$_{54}$ molecules.
The derivatives of the wavenumber-temperature curves (Figure 4.22) reveal well the transition temperature ranges of the single components. From these curves it can be seen that the DMPC component of DMPC-d$_{54}$/DPPG/PLA complexes melts in three steps. The same behaviour was observed by DSC experiments (Figure 4.3). However, DSC is only able to detect the summation of the effects originating from both membrane constituents. With the help of the IR experiments, the three step melting behaviour can be assigned to the DMPC component.

**Secondary structure of PLA bound to mixed PC/PA membranes**

As has been shown for the DPPG/DPPC/PLL complexes, domain formation in mixed membranes can trigger secondary structure changes of the bound peptide. Therefore, we examined also the secondary structure of PLA bound to a mixed DPPC/DPPG membrane at different temperatures. Indeed, it can be seen that the secondary structure differs from that PLA adopts upon binding to pure DPPG membranes (Figure 4.23). Second derivative spectra reveal that the positions of the amide I components differ from those that were described above. In principle, two components are found at 1634 cm$^{-1}$ and 1645 cm$^{-1}$. At higher temperatures, a further band develops at 1653 cm$^{-1}$. In addition, turn structures that give rise to a vibrational band at about 1670 cm$^{-1}$ are present in all the spectra. Contrary to the spectra of PLA bound to pure DPPG, no $\beta$-sheet vibrations are found. The bands that are found in the spectra originate from $\alpha$-helix and coil structures. The difficulties of an exact assignment have been discussed above. Interesting is the temperature dependent shift of the bands. In contrast to the case of PLA binding to pure DPPG, the band intensities do not change continuously over the whole temperature range. Rather, the band that is located at 1635 cm$^{-1}$ shifts at $T_m$ of the mixed membrane to higher wavenumbers (Figure 4.23c). This shows that the phase transition of the lipid induces a secondary structure change of the bound peptide. The phase transition of the lipid acyl chains and the bound peptide start at the same temperature. However, the temperature range of the transition is broader for the peptide than it is for the acyl chains. Probably, the peptide secondary structure transition follows the phase transition of the membrane.

These findings are very similar to what was found for PLL complexes with mixed DPPC/DPPG membranes and can be explained in the same manner. The charge density of the gel phase domains is higher than that of the remixed liquid crystalline membrane. Only if the charge density is high, the side chain charges of the peptide are electrostatically screened, allowing the peptide to adopt a secondary structure. But probably the charge density is not high enough for the formation of a $\beta$-sheet, even in the gel state domain with increased DMPA content.
Figure 4.23: a: Amide I and guanidyl stretching vibrational bands of PLA 649 bound to DPPC/DPPG mixed membranes at different temperatures. b: Second derivatives of curves presented in a in the region of the amide I band. c: Temperature dependence of the amide I band components as revealed by the second derivatives (○) and the frequencies of νs(CH2) (■).

4.6 Fluorescence experiments

The experiments described before show that PLA interacts not only superficially with PG bilayers, but rather inserts to some extent into the headgroup and interfacial region. The question arises how much the bilayer integrity is affected by the interaction with the polypeptide. Therefore, dye release experiments were performed. A fluorescence dye in self quenching concentration (80 mM calcein) was included in the aqueous interior of vesicles. As long as the barrier function of the vesicles membrane is retained, no fluorescence can be detected. If, the membrane integrity is disturbed, dye can be released from the vesicle into the bulk solution, were due to the dilution, it is not any longer self quenching. Thus, the fluorescence intensity rises, as the membrane gets porous or vesicles are ruptured. ITC and DSC experiments showed that PLA binding to fluid PG membranes saturates at concentrations where, assuming a 1:1 stoichiometry, also lipids of the inner lamella have to interact with the peptide. Therefore, PLA might either be translocated through permanent or transient pores that are induced by PLA binding, or the vesicles disrupt. In both cases the membrane structure will be disturbed and enclosed dye can efflux.
Figure 4.24: Kinetics of dye release from POPG vesicles after interaction with PLA 69. The POPG/Arg mixing ratios ($R_c$) are (—) 7.69; (—) 2.6; (—) 1.56; (—) 0.78; (—) 0.26; (—) 0.16. The curves are normalized to the dye release from POPG vesicles induced by addition of TRITON X. The dye release is given in percent of this maximal value.

An example of dye release kinetics is shown in Figure 4.24. It can be seen that PLA induces dye release from fluid state POPG vesicles only at certain concentrations. If excess PLA concentrations or excess lipid concentrations are used, the bilayer integrity is not affected. However, PLA induces dye efflux, if added in the concentration that leads to neutralization of the outer vesicle monolayer ($R_c = 2$). This finding was confirmed for all examined systems, independent of the peptide chain length and the membrane composition. This implies that a prerequisite for pore formation is charge neutralization of the outer monolayer by PLA. A neutral PG/PLA complex might then overcome the hydrophobic barrier and form transient pores. These pores might be the pathway for peptide translocation as well as for dye efflux. Such an model was also proposed by Tang et al. (2007), who postulated on the basis of NMR experiments that phospholipid/arginine complexes form toroidal pores in PE/PC membranes.

The fact that the dye is not completely released indicates that the vesicle structure is not destroyed and that pores or defects that allow the dye to escape from the vesicle interior are only transient. This is supported by cryo-TEM images, which show that the vesicle structure is retained even if aggregates are formed (Figure 4.25).
If the experiments are performed with DPPG gel state vesicles the maximal dye release is reduced (Figure 4.26). This shows that the bilayer structure in the gel phase is not as strongly perturbed by PLA binding as in the liquid crystalline phase. This indicates that “fluid” lipid molecules are necessary for pore formation. The dye efflux is further reduced when zwitterionic PC is admixed to gel phase or fluid phase membranes. This effect can be attributed to the reduced electrostatic attraction and to the fact that the uncomplexed and mobile PC molecules might compensate membrane defects that are induced by peptide adsorption.

Very similar trends are also observed for PLL binding to PG containing membranes. However, the absolute values of PLL induced dye release are in all cases lower than those detected in comparable PLA experiments (Figure 4.26). This confirms the proposition given in the foregoing chapters. PLL interacts more electrostatically with PG membranes than PLA and hydrophobic as well as hydrogen bonding interactions that lead to peptide insertion are less important for PLL binding than for PLA binding.
4.7 Summary

In this chapter the binding behaviour of the positively charged polypeptide PLA to negatively charged DPPG membranes was studied as a function of PLA chain length, the lipid to peptide mixing ratio $R_c$ and the phase state of the membrane.

**Thermotropic phase behaviour**

DSC experiments revealed that the influence of PLA binding on the main phase transition temperature $T_m$ of DPPG membranes is unexpectedly small. $T_m$ is shifted to slightly lower or higher values dependent on the chain length of the binding peptide. This is attributed to the presence of different binding processes that are compensating each other. The cooperativity of the main phase transition is decreased in lipid excess complexes. In contrast, the cooperativity is increased in peptide excess complexes. This indicates that well defined PLA/DPPG complexes are formed.

**Binding thermodynamics**

To prove the existence and reveal the nature of different binding processes we performed ITC and monolayer experiments. ITC revealed indeed at least two different processes in PLA binding to gel state membranes, one of them being endothermic and the other being
exothermic. Moreover, it was found that the binding to fluid phase membranes is energetically favoured. This could be deduced from the more negative binding enthalpies $\Delta_R H$ and the higher binding constants $K$ in case of PLA binding to fluid membranes as compared to gel state membranes. Longer PLAs bind more exothermic and with higher binding constants than shorter ones.

**Hydrophobic interactions**

Besides electrostatic also hydrophobic interactions contribute to PLA binding, as shown by temperature dependent ITC measurements on fluid POPG membranes. The determined negative $\Delta_R C_p$ is indicative for the release of water molecules from hydrophobic surfaces. Thus, it can be assumed that hydrophobic parts of the arginyl side chain get buried in the membrane during the binding process. By monolayer adsorption and dye release experiments could be shown, that indeed the binding is not only superficial but that moieties of the polypeptide insert into a DPPG membrane.

**Monolayer adsorption**

Monolayer experiments provide evidence for at least two consecutive binding processes. The peptide has a condensating effect on monolayers of low initial pressure $\pi_0$ (LE phase). This condensation is reflected by a surface pressure decrease ($\Delta \pi < 0$). It is followed by an insertion of the peptide or parts of the peptide into the monolayer, which results in a final surface pressure increase ($\Delta \pi > 0$). Monolayers of high $\pi_0$ (LC phase) are not condensed any more but still penetrated by the peptide. A surprising result is that $\Delta \pi$ increases with increasing $\pi_0$. This could be attributed to a difference in compressibility of the free and the bound monolayer and the high peptide exclusion pressures. This shows that the lipid monolayer strongly interacts with the inserted peptide.

**Interfacial hydration**

Structural information on the organisation of the DPPG/PLA complexes could be derived from FT-IR experiments. The analysis of the C=O stretching vibrational bands showed that PLA binding releases water molecules from the interfacial membrane region. Especially in the fluid crystalline phase, hydration of the carbonyl groups is reduced by PLA binding. This supports the results of ITC and monolayer experiments, which predict water release and side chain insertion. However, the remaining water molecules are bound by stronger hydrogen bonds. The water molecules are better oriented and restricted in their reorientational motion.
Acyl chain order

Lipid molecules are also restricted in their rotational dynamics. This could be deduced from the increased interchain coupling of the methylene dipoles, which reduces the frequencies of the CH$_2$ stretching vibrations. It could be demonstrated by isotopic dilution experiments that these coupling effects have to be taken into account, if CH$_2$ stretching vibrational frequencies are interpreted.

Secondary structure

The amide I vibrational frequencies of PLA in different secondary structures were analysed. The results were used to identify the structure PLA adopts upon binding to DPPG membranes. It was shown that longer PLAs bind in a β-sheet conformation to gel phase membranes. This β-sheet unfolds to a random coil when the temperature is raised. Shorter PLAs do not form β-sheets upon binding. The reduction of the membranes charge density by co-addition of zwitterionic DPPC prevents the formation of β-sheets. The existence of α-helical structures at higher temperatures is proposed but cannot be proven on the basis of the performed experiments.

Lipid demixing

DSC and IR experiments with mixed PC/PG membranes showed that PLA induces domain formation in gel phase PC/PG membranes. Upon PLA binding a PG enriched binding domain is formed. The phase diagram shows a very wide and asymmetric gel-liquid crystalline coexistence region.

Binding of PLA to negatively charged PG containing membranes shows typical electrostatic features but has also noticeable non electrostatic contributions. This confirms the results given by Hitz et al. (2006) and Goncalves et al. (2005). The non electrostatic contribution arises from hydrophobic interactions of the arginyl side chain with the interfacial membrane layer. But also hydrogen bonding might be involved which was already reported to play a key role in arginine interaction with membrane lipids (Rothbard et al. 2004; Rothbard et al. 2005; Tang et al. 2007).
4.8 Comparison of PLL and PLA binding to PG membranes

Although PLL and PLA are very similar in structure and charge (see chapter 2.2), their binding behaviour is remarkably different. These differences can only be explained if other than electrostatic interactions are involved. Comparison of the binding behaviour and the complex properties is very instructive to reveal the specificities of the two amino acids lysine and arginine. Differences and similarities in the interaction of PLL and PLA with PG containing membranes will be shortly summarized in the following paragraphs.

Transition temperature

PLL and PLA influence the gel to liquid crystalline phase transition of DPPG membranes differently. Whereas PLL increases $T_m$ by about 4–5 °C, PLA has nearly no effect on $T_m$. The influence of PLL is well explainable with electrostatic interaction at the membrane surface, which stabilizes the gel phase of the membranes. The transition behaviour of the PLA/DPPG complex can only be explained if non electrostatic interactions compensate for the electrostatic effect.

Domain formation

PLL as well as PLA binding to mixed PC/PG membranes leads to reduced miscibility of the membrane lipids. Domains with increased PG content are formed to provide a high electrostatic surface potential for peptide binding. PLL binding leads to a macroscopic miscibility gap in gel phase membranes if the PG content is lower than 50%. There are also indications for fluid-fluid demixing for membranes with higher PG content (60–80%).

PLA binding influences the PC-PG phase diagram in a similar way, but the effects are less pronounced. Possibly, PLA binding also leads to gel phase demixing in membranes with low PG content. In contrast, fluid demixing is not observed for PLA binding.

The reason for domain formation is the favourable electrostatic interaction of the peptides with highly charged domains. The fact that demixing is more obvious for PLL binding shows that electrostatic interactions are more important for PLL binding than for PLA binding.

Binding enthalpies

ITC studies revealed substantial differences in PLL and PLA binding to PG containing membranes. While PLL interacts with an exothermic $\Delta H$ with gel phase membranes and nearly no enthalpic contribution for fluid state binding, PLA behaves exactly opposite. PLA binding to fluid membranes is clearly exothermic, whereas the total heat released upon PLA binding to gel phase membranes is nearly zero. However, from the titration curves it can be seen that exo-
and endothermic processes are present, but compensate each other. The negative heat capacity change during the reaction ($\Delta R C_p < 0$) implies that hydrophobic interactions play a role in both, PLL and PLA binding. However, $\Delta R C_p$ is more negative in the case of PLA binding, which can be attributed to a higher hydrophobic contribution to the binding process.

**Hydration of the membrane interface**

The different interaction modes of PLL and PLA with membranes in different phase states are reflected by their different influence on the hydration of the interfacial carbonyl groups. Gel phase membranes are better hydrated if PLL is bound, while PLA binding does not influence gel phase hydration. In contrast, PLA binding dehydrates the carbonyl group of membranes in the liquid crystalline phase, whereas PLL binding has no influence on the carbonyl hydration in this phase state.

Probably, inserted PLA side chains replace water molecules from the interfacial region. Remaining water molecules have to be shared by the guanidyl group and the lipid carbonyl group. Guanidinium competes much more with the carbonyls for hydration water than the lysine groups do, because of its higher polarity and the presence of more hydrogen bonding sites. In bulk solution one arginine side chain binds six water molecules, whereas one lysine side chain binds only two (Collantes and Dunn 1995). PLL probably entraps water molecules in the inter-headgroup space without competing for them. The dehydrating effect of PLA binding is reflected by a tendency of the membrane to form recrystallized phases before acyl chain melting (Förster et al., unpublished results). PLL, by contrast, which increases the hydration of the gel phase and does not affect the hydration of the fluid phase, suppresses any tendency for recrystallization.

**Acyl chain order**

The methylene stretching vibrational frequencies decrease upon PLL and PLA binding, which is normally interpreted with an increase of the acyl chain order. However, it could be shown that this effect is mainly due to increased interchain vibrational coupling. If vibrations of neighbouring acyl chains are decoupled by isotopic dilution (i.e. partial deuteration), the effect of decreasing $\nu(\text{CH}_2)$ frequencies vanishes nearly completely in the case of PLA binding. In the case of PLL binding the effect gets smaller, but a decrease of the $\nu(\text{CH}_2)$ frequencies is still encountered, even when the vibrations are decoupled. This remaining decrease is attributed to acyl chain ordering. This leads to the conclusion that PLL binding has a higher ordering effect on the lipid acyl chains than PLA binding.
Secondary structure

PLL and PLA adopt different secondary structures upon binding to PG membranes. PLL forms a $\alpha$-helix on the surface of DPPG gel phase bilayers. At temperatures higher than $T_m$ the helix gradually unfolds. Prerequisite for helix formation upon binding is a sufficiently high surface potential of the membrane. If the surface potential is lowered by admixing of zwitterionic lipids at conditions were no demixing occurs, no helix will be formed.

In contrast, PLA adopts a $\beta$-sheet conformation upon binding to gel state DPPG membranes. Temperature increase and admixing of zwitterionic lipids have similar effects on the $\beta$-sheet as on the $\alpha$-helix in the case of PLL binding. However, after unfolding of the $\beta$-sheet the structure is not pure random coil. Rather another secondary structure element is preserved. This might be due to a conformation of inserted peptide moieties, which differs from the secondary structure the peptide adopts upon adsorption to the surface.

Monolayer adsorption

In principle PLL and PLA have the same effects on a DPPG monolayer. An expanded monolayer (LE phase) condenses after peptide adsorption. The condensation is followed by insertion of peptide side chains in the condensed domains. Both peptides insert in condensed monolayers (LC phase). This shows that hydrophobic interactions play a role in both, PLL and PLA binding. However, the condensating effect is more pronounced in the case of PLL adsorption, whereas insertion is more evident in the case of PLA adsorption. This shows again that electrostatics interactions are more important for PLL binding, whereas hydrophobic interactions are more prominent in PLA binding.

Dye release

Both, PLA and PLL induce dye release from fluid POPG vesicles when added in an amount that compensates the outer monolayer charges. However, dye release is much higher in the case of PLA binding than in the case of PLL binding. This confirms the conclusions drawn from monolayer adsorption experiments. PLA inserts more deeply into the lipid bilayer and induces defects and/or pore formation. The reason is its higher propensity to form stable charge compensated complexes with the phospholipid headgroup. Higher hydrophobic interactions lead then to insertion into the bilayer. Lower hydrophobicity and remaining charge density prevents PLL from inserting to the same extent.

Chain length dependencies

All described effects are dependent on the chain length of the polypeptide. In general the effects are more pronounced, if the polypeptide is longer. This dependency is valid for both, PLL and PLA. In the case of PLL binding the chain length dependencies were investigated in
more detail. It was shown that the chain length dependency reverses at a certain length. Here steric constraints compete with thermodynamic effects. The shortest PLL \((n = 14)\) behaves in many aspects differently than predicted from the chain length dependencies. This distinct behaviour could be attributed to its inability to form a defined secondary structure.