Physico-Chemical Investigations of, and Characterization of ModelMembranes for, Lipid-Peptide Interactions

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Dissertation presented at Uppsala University to be publicly examined in B22, BMC, Husargatan 3, Uppsala, Friday, March 27, 2009 at 09:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

The main focus of this thesis is on the effects caused by α-helical peptides on liposome structure, the impact of cholesterol on the peptide-membrane interactions, and the effect of aggregate curvature on the peptide affinity. Results of the studies show that the membrane destabilizing effect of the cationic α-helical peptide melittin is modulated by cholesterol. Melittin induces leakage from pure phospholipid liposomes in a manner that is compatible with the presence of small pores. In the case of cholesterol-supplemented liposomes leakage coincides, however, with major structural transformations and rupture of the liposomes. Cholesterol decreases the affinity of melittin for phospholipid bilayers, but once the peptide has adsorbed to the membrane the presence of cholesterol does not offer any resistance against melittin-induced membrane destabilization. Our investigations indicate that cholesterol affects the alamethicin-lipid bilayer interactions in a similar way.

PEG-stabilized bilayer disks are formed upon addition of polyethylene glycol (PEG)-lipids to lipid mixtures with high bending rigidity. The partial segregation of components within the bilayer disk, suggested by theoretical calculations and experimental observations, was verified by small angle neutron scattering. By means of a novel competitive binding assay it was shown that the three α-helical peptides melittin, alamethicin, and magainin have high affinity for the curved rim of PEG-stabilized bilayer disks. The bilayer disks have structural, and other properties, that make them interesting for the formulation of peptides and membrane-associated proteins. For stability reasons dry formulations are often preferred. The PEG-stabilized bilayer disks were shown to retain their structure in rehydrated samples that had been freeze- or spray-dried in the presence of lactose.

Keywords: lipid-bilayer, liposome, melittin, cryo-TEM, neutron scattering, dynamic light scattering, spray-drying, freeze-drying, alfa-helical peptide, disk, cholesterol, alamethicin.

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ISSN 1651-6214
urn:nbn:se:uu:diva-89432 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-89432)
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   Per Wessman, Adam A. Strömstedt, Martin Malmsten, Katarina Edwards
   *Biophysical Journal, 2008, Vol. 95, pp 4324-4336*

II  Effect of Lipid Headgroup Composition on the Interaction between Melittin and Lipid Bilayers
   Adam A. Strömstedt, Per Wessman, Lovisa Ringstad, Katarina Edwards, Martin Malmsten

III  Effect of $\alpha$-helical Peptides on Liposome Structure: a Comparative Study of Melittin and Alamethicin
    Per Wessman, Malin Morin, Karin Reijmar, Katarina Edwards
    *Manuscript*

IV  Melittin-Lipid Interaction: A Comparative Study Using Liposomes, Micelles and Bilayer Disks
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    *Biochimica et Biophysica Acta, 2008, Vol. 1778, pp 2210-2216*

V   Structural Effects Caused by Spray and Freeze Drying of Liposomes and Bilayer Disks
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Abbreviations

\(a_0\)  Effective head group area
CF  Carboxyfluorescein
DLS  Dynamic light scattering
DOPA  \(1,2\text{-dioleyl-}sn\text{-glycero-3-phosphatidic acid}\)
DOPC  \(1,2\text{-dioleyl-}sn\text{-glycero-3-phosphatidylcholine}\)
DOPE  \(1,2\text{-dioleyl-}sn\text{-glycero-3-phosphatidylethanolamine}\)
DPG  diphosphatidylglycerol
DPPC  \(1,2\text{-dipalmitoyl-}sn\text{-glycero-3-phosphatidylcholine}\)
DSC  Differential scanning calorimetry
DSPC  \(1,2\text{-distearoyl-}sn\text{-glycero-3-phosphatidylcholine}\)
E-coli  Escherichia coli
EPC  Egg phosphatidylcholine
FBM  Fluorescence based method
\(K_A\)  Area compression/expansion modulus
\(k_C\)  Bilayer bending rigidity
\(K_p\)  Partitioning coefficient
\(l\)  Length of the hydrocarbon chains
\(L_C\)  Solid crystalline phase
\(l_o\)  Liquid ordered phase
\(L_\alpha\)  Liquid crystalline phase
\(L_\beta\)  Gel phase
\(N_{CPP}\)  Critical packing parameter
PC  Phosphatidylcholine
PE  Phosphatidylethanolamine
PEG  Polyethylene glycol
PG  Phosphatidylglycerol
PI  Phosphatidylinositol
POPC  \(1\text{-palmitoyl-2-oleyl-}sn\text{-glycero-3-phosphatidylcholine}\)
POPG  \(1\text{-palmitoyl-2-oleyl-}sn\text{-glycero-3-phosphatidylglycerol}\)
PS  Phosphatidylserine
\(R_{eff}\)  Molar ratio of membrane associated melittin to lipid
\(R_h\)  Hydrodynamic radius
\(R_i\)  Lipid mixing ratios
SANS  Small angle neutron scattering
SDS  Sodium dodecyl sulphate
SM  Sphingomyelin
TEM  Transmission electron microscopy
\( T_g \)  Glass transition temperature
\( T_m \)  Main transition temperature
\( v \)  Volume of the hydrophobic tail
\( w \)  Parameter describing deviation from ideal partitioning
\( w/v \)  Weight by volume
Amphiphilic molecules and self-assembly

Amphiphilic molecules have both a “solvent liking” lyophilic and a “solvent hating” lyophobic part. If water is used as the solvent one talks about the hydrophilic polar part and the hydrophobic apolar part. When dispersed in aqueous media the latter interact unfavorably with the water. Because of this dual character amphiphilic molecules have a preference for interfaces between polar, e.g. water, and apolar media, e.g. oil or air. Amphiphilic molecules are therefore often also referred to as surfactants (short for surface active agents). Figure 1 show a schematic drawing of a conventional surfactant in which the hydrophilic area is termed the headgroup and the hydrophobic part the tail.

![Figure 1. A schematic illustration of a conventional surfactant.](image)

At low concentrations amphiphiles are dissolved as monomers. However, at a critical concentration they will assemble into molecular aggregates in a fashion that shields the hydrophobic part of the amphiphile from the surrounding water. The driving force for self-assembly is the so-called hydrophobic effect which is of entropic origin (1, 2). Water molecules can not form hydrogen bonds with the apolar molecules. Therefore, water in the vicinity of the hydrophobic part of the amphiphile will be structured as to retain as many hydrogen bonds as possible. The self-assembly of the amphiphiles imposes less structure on the water and is therefore energetically more favorable for the system.
Geometry of self-assembled aggregates

Different type of aggregates will form depending on the molecular geometry of the surfactants. The spherical micelle is the simplest aggregate assembled by molecules with a large head group and one hydrocarbon chain, e.g., Sodium dodecyl sulphate (SDS) which is often used in soaps and tooth paste. A change in molecular shape will infer a change in aggregate structure. For instance, increasing the volume of the tail makes it harder to assemble the surfactants in a spherical shape. Instead the micelle will grow and become rod like.

A way of predicting what type of structure a certain surfactant will form, based on the shape of the molecule, is to use the concept of the critical packing parameter $N_{CPP}$ (2),

$$N_{CPP} = \frac{v}{l \times a_0}$$

where $v$ is the volume of the hydrophobic tail, $l$ is the length of the hydrocarbon chains of the tail and $a_0$ is the effective headgroup area. Figure 2 gives an overview of the type of aggregates that are formed for different molecular geometries and $N_{CPP}$. The advantage of this model is its simplicity and relative accuracy for pure surfactants. However, there are downsides with the concept of the critical packing parameter as well. The effective headgroup area can be difficult to predict since it often depends on the physico-chemical properties of the surrounding such as electrolyte concentration, pH and sometimes temperature. It may also, in it self, depend on the aggregate curvature (3). Moreover, aggregate structures that are not predicted by the concept of the critical packing parameter have been found (4).

Another way of predicting the geometry of self assembled aggregates is to look at the curvature of an unstrained monolayer of the surfactant molecules (5). If the monolayer curls around the hydrophobic part, as for micellar-forming surfactants, the spontaneous curvature is defined as positive. A negative spontaneous curvature as observed for surfactants with a large hydrophobic volume and/or a small head group area leads to the formation of inverted structures. Molecules that form planar monolayers have near zero spontaneous curvature and will assemble into lamellar structures (6).

The lamellar phase

Upon increasing the surfactant concentration a variety of different liquid crystalline phases are formed depending on the nature of the surfactant molecule. The appearance of these phases can be envisaged by assuming close packing of the aggregates, as shown in Figure 2.
Amphiphiles with a cylindrical geometry, i.e. having a $N_{cpp}$ close to one, are packed into bilayers or lamella. Stacks of bilayers build up the biologically important lamellar phase. Hydration of the lamellar phase leads to an increase in the bilayer repeat distance as water now interspaces the lamellae.

Cell membranes can be considered as a lamellar phase dispersed in excess water. The reason that the cell membrane does not disintegrate in aqueous surroundings is the low monomer solubility of it’s building blocks, for example the phospholipids discussed below. Their monomer solubilities are often as low as $\sim 10^{-10}$ M and therefore lipid bilayers will not dissolve in excess water unless extremely diluted.
Phospholipid bilayers

The glycerophospholipids, or phospholipids, constitute the major part of the lipids in cell membranes. The head group involves of a phosphate linked to the glycerol backbone in the 3-position and the tail normally consists of two hydrocarbon chains in the 1- and 2-position. Different amine or sugar groups can be linked to the phosphate giving the head group different properties. Figure 3 shows the structures of the phospholipids used in this work. The length and degree of saturation of the acyl, i.e. hydrocarbon, chains can also be varied. However, gross classification of the phospholipids is based on the head group structure.

**Figure 3.** The molecular structure of the phospholipids used in the present work. The hydrocarbon chains (left) are linked to the glycerol backbone (middle). The different headgroups are shown to the right. The structure of the hydrocarbon chains are indicated by the denotation $C_{X:Y}$ where $X$ is the length of the hydrocarbon chain and $Y$ is the number of unsaturations in the chain.

Most of the phospholipids spontaneously form bilayers under normal conditions but variation in pH, temperature and the aqueous electrolyte concentrations may affect the curvature of the lipids and promote inverted structures. The spontaneous curvature of the lipids is also dependent on acyl saturation as cis double bonds will introduce kinks in the hydrocarbon chain affecting the volume of the hydrophobic tail. Phospholipids with only one acyl chain, the so-called lysophospholipids, form micellar structures.
Phase behavior and micromechanical properties of phospholipid bilayers

Phospholipid bilayers display both thermotropic and lyotropic phase behaviour. This section will focus on the effect of temperature on fully hydrated bilayers. Several solid-like and one fluid phase have been characterized for phospholipid bilayers. These are, in order of increasing temperatures, the solid crystalline phase, $L_C$, the gel phase, $L_{eta}$, and the liquid crystalline phase, $L_a$. The $L_{eta}$ phase is characterized by well ordered acyl chains in a predominantly all-trans conformation and slow lateral lipid diffusion. Significant for the PC-bilayers in the $L_{eta}$ phase are the tilted head groups. Cooperative chain melting takes place at the main transition temperature, i.e. changing from a solid to a liquid-like phase. The acyl chains are now disordered and rapid trans-gauche isomerization takes place. Furthermore, phospholipids in the $L_a$-phase exhibit rapid lateral diffusion. The main transition temperature, $T_m$, increases with the length of the hydrocarbon chains and is lower for an unsaturated phospholipid than for its saturated equivalent (4, 7).

The material properties of phospholipid bilayers can be characterized by the work needed to be done on the bilayer in order to bend it or to change the area per lipid molecule in the bilayer. These energies are proportional to the bilayer bending rigidity, $k_C$, and area compression/expansion modulus, $K_A$, respectively (8-10). For PC bilayers with only one lipid component $k_C$ increases with chain length whereas $K_A$ varies little regardless of chain length or degree of unsaturation (10).

Cholesterol in phospholipid bilayers

Beside the phospholipids the sterols are among the most abundant lipids found in many cell membranes. Cholesterol is found in animal membranes and has a pronounced effect on the membrane properties of phospholipid bilayers. The structure of cholesterol is distinguished by the small hydroxyl head group and the large hydrophobic part made up by the rigid ring structure and the alkyl side chain, see figure 4.

![Figure 4. The molecular structure of cholesterol](image)

The head is believed to reside close to the ester carbonyl of the PC head group while the ring structure interacts with the acyl chains of the lipids. The alkyl chain extends into the center of the bilayer (11). Addition of cholesterol...

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1 Depending on the type of phospholipid different gel mesophases have been characterized.
terol to PC-bilayers in the liquid crystalline phase induces an ordering of the lipid chains whereas the order decreases for lipids in gel phase bilayers. The main phase transition is thus reduced as a result of increasing the amount of cholesterol in the bilayers and at concentrations above 20 mol% a new bilayer phase appears, termed the liquid ordered phase and denoted $l_o$ (12, 13). Apart from the cholesterol concentration, the onset of the $l_o$ phase also depends on lipid chain saturation and temperature (14-17). As suggested by the terminology PC-bilayers in the liquid ordered phase retain, despite the chain ordering effect, a liquid-like behavior that is characterized by a relatively high in-plane diffusion. Other important effects of cholesterol on PC-bilayers are the stiffening of the membranes, expressed as an increase of the bending and the area compressibility modulus, and reduction of the permeability of the membranes (18-21).

### Biological cell membranes

Common to all living organisms is the plasma membrane that defines the cell boundary. Complex cells such as the eucaryotic cell also have intracellular membranes, defining different organelles, whereas the simpler prokaryotic cell lacks membrane-defined inner cell compartments. As mentioned earlier the bilayer framework of the cell membranes is made up of lipids with phospholipids as a major component. Apart from the lipids as much as half the weight of the plasma membrane is made up of proteins. A fundamental function of the plasma membrane is as a barrier that allows the cell to maintain a chemical gradient of ions and solutes. The loss of the chemical gradient inevitably leads to cell death (22).

The lipid composition of plasma membranes varies much, not only between different species but also for different types of tissues. As an example of an eucaryotic plasma membrane the lipid composition of the human erythrocyte is 45% cholesterol, 17% PC, 17% sphingomyelin (SM), 16% phosphatidylethanolamine (PE), all zwitterionic at neutral pH, and 6% of the negatively charged phosphatidylserine (PS) (22). However, other lipids such as phosphatidylinositol (PI) and glycolipids can also be found. Further, a common feature, at least for eucaryotic cells, is the asymmetrical distribution of the lipid composition between the inner and outer leaflet of the plasma membrane bilayer (23, 24). For eucaryotic cells this asymmetry is manifested by the fact that nearly all negatively charged lipids in the plasma membrane are located in the inner leaflet of the plasma membrane facing the cytoplasm (25).

Bacteria or prokaryotic plasma membranes differ from eucaryotic ones in that they contain a substantial amount of negatively charged lipids and do not contain any cholesterol. The lipid composition of the plasma membrane of *E-coli* is dominated by zwitterionic PE and negatively charged phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). Gram-negative bacteria
such as *E-coli* also contain an outer membrane. The outer leaflet consists of negatively charged lipopolysaccharides and the inner leaflet contains PE, PG and DPG (4, 26). For a schematic illustration of the eucaryotic and prokaryotic cell membranes see Figure 5.

![Figure 5](image.png)

*Figure 5.* A schematic illustration of the eukaryotic (top) and the prokaryotic cell membrane (bottom). The eukaryotic membrane is distinguished by the presence of cholesterol as well as the asymmetrical location of anionic lipids. The prokaryotic inner membrane contains high amount of anionic lipids but no cholesterol.

**Antimicrobial peptides**

Peptides with membrane disruptive effect are part of the innate defense system of many different animal and plant species. They represent an evolutionary ancient weapon against bacteria, and sometimes fungi. The cell recognition is based on properties that distinguish the microbial bilayer from the host membranes, such as charge density and lack of cholesterol (27, 28). Adsorbed to the microbial cell surface the peptides permeabilize the membrane and thereby destroy the barrier properties crucial for sustaining the viability of the cell. However, the lethal effect of some peptides may involve intracellular action rather than membrane lysis. The more than 800 antimicrobial peptides that have been characterized so far show great structural diversity. The peptides are broadly classified on the basis of their secondary structure, charge and/or the predominance of certain amino acids (28, 29). The linear and amphiphilic α-helical peptides comprise an important group of the antimicrobial peptides. By modifying the amino acid sequence it is possible to design new antimicrobial peptides. The aim of such modifications is to increase the antimicrobial specificity of the peptides and reduce toxic effects.
The action of linear $\alpha$-helical peptides

The association of the $\alpha$-helical peptides to lipid bilayers is driven by hydrophobic interactions with the hydrocarbon chains of the lipids, electrostatic interactions between charged peptides and oppositely charged lipids and the folding of the peptide into a $\alpha$-helical conformation. When free in solution the peptides are largely unstructured. As the peptides adsorb to the bilayer surface they fold into an amphiphilic $\alpha$-helix with one hydrophobic and one hydrophilic surface (30-32). The peptides align to the outer bilayer leaflet parallel to the membrane with the hydrophobic surface of the helix facing the bilayer core. This allows the hydrophobic side chains of the peptide to be buried in the lipophilic part of the bilayer and the hydrophilic side chains to remain in the polar environment of the lipid head groups region. Mechanical properties of the lipid bilayers will influence the ability of peptides to associate with the membranes (33, 34). For example, inclusion of cholesterol in the lipid bilayers tends to decrease the ability of peptides to associate with the membranes (35, 36).

In order to accommodate it the lipids have to rearrange in the proximity of the adsorbed peptide, resulting in an area expansion as well as a membrane thinning (37-39). The mass imbalance due to the adsorption of peptides to the outer leaflet induces a positive curvature strain on the bilayer. The stress is believed to be relieved by the formation of pores in the membranes which allows for a transport of lipids and peptides between the leaflets (27, 40). Bilayer properties such as spontaneous curvature, bending rigidity and area compressibility modulus affect the permeabilizing ability of the peptides (33, 35, 41, 42).

The creation of the rim of the pore is associated with an increase in free energy. To shield the exposed hydrophobic area at the rim, the bilayer-forming lipids rearrange so that their lipid head groups line the pore. Exposed hydrophobic surfaces and strains imposed on the lipids participating in the pore results in the creation of an edge tension that will act to close the pore. Surfactants and amphiphilic peptides have been shown to preferentially bind to the rim and can act to lower the edge tension thereby promoting pore formation. (43, 44) Cholesterol tends to increase the edge tension (43).

Although there now seems to be a consensus regarding peptide adsorption the mechanism for membrane disruption is still under debate (45, 46). Likely, different mechanisms apply to different peptides. Moreover, the disrupting mechanism appears to be dependent on bilayer composition (47, 48). Theories trying to unify different experimental results have, however, been suggested (27, 29, 46, 49). The peptides adsorb to the outer leaflet and at a certain peptide to lipid ratio pores are formed as a response of peptide induced membrane stress. Several different pore structures have been suggested based on experimental data. Two well defined structures are the toroidal and the barrel stave pores. The toroidal pore is often described as a supra-aggregate of peptides and lipids. The membrane bends so that the pore
is lined with lipid headgroups and peptides. In contrast, the channel in the barrel stave pore is lined solely by peptides. There are experimental data supporting both structures (33, 50, 51). Other less well defined pore structures have also been proposed (52). At higher peptide concentrations the membrane collapses completely (27, 28, 46, 49). In the “detergent-like mechanism” defects or openings in the membrane form transiently at low peptide concentrations, however not as well organized as suggested for the toroidal pore. Micellization takes place at high peptide to lipid ratios (45). Highly organized pores such as the toroidal pore or the barrel stave pore require that the peptides reorient from a surface parallel to a perpendicular orientation. This reorientation has been observed for several peptides and appears to take place at certain peptide to lipid ratios depending on peptide and bilayer composition (41). It is suggested that the reorientation marks the onset for pore formation. However, how well correlated the reorientation is to the permeabilization of membranes is still not clear.

Some of the most studied α-helical peptides are melittin (53, 54), found in bee venom, the magainins (27), isolated from the skin of an African frog, and alamethicin isolated from a fungus (55). They exemplify both the similarity and the diversity in structure and action of the α-helical peptides. A common feature is of course that all three peptides take on an linear amphiphilic α-helical structure when associated with lipid bilayers. However, whereas melittin and the magainins are cationic alamethicin distinguish itself by being practically uncharged at physiological conditions. See Table 1 for an overview of the amino acid sequences. Moreover, whereas the magainins show specificity for bacterial membranes, alamethicin and melittin also have a pronounced haemolytic effect (27, 55, 56). The studies reported in Paper I – IV concern the effect of melittin on various lipid bilayers. Paper III also focus on some aspects of the interaction of magainin 2 and alamethicin with lipid structures.

Table 1. Amino acid sequences of melittin, magainin 2 and alamethicin. The cationic amino acid residues are marked bold and the anionic glutamate in magainin 2 is italic.

| GlylleGlyAlaVal LeuLysValLeuThr ThrGlyLeuProAla LeuIleSerTrpIle LysArgLysArgGln Gln-CONH2, Melittin |
| GlylleGlyLysPhe LeuHisSerAlaLys LysPheGlyLysAla PheValGlyGluLele MetAsnSer-CONH2, Magainin 2 |
| AsnAlaValProAlbAlb AlaGlnAlbValAlb GlyLeuAlbProVal AlbAlbGlnGlnPhe-CONH2, Alamethicin |

The formation of toroidal pores have been experimentally proven at high peptide to lipid ratios for melittin (51). On the other hand detergent-like mechanism of melittin have also been reported (47). Also, for the magainins and alamethicin, the respective epitomes for peptides that form toroidal and barrel stave pores, the pore structure is debated (50, 52, 57, 58). The pores formed do not necessarily need to be lined with peptides (40).
Lipid-based nano-particles: liposomes and disks

Liposomes

Self-closed structures of lipid bilayers that encapsulate part of the medium that they are dispersed in are called liposomes, see Figure 6. They may consist of one or several bilayers and have diameters from 20 nm up to several micrometers. In liposome-based research it is common to use unilamellar liposomes, i.e. liposomes having one bilayer, with radii of around 100 nm. However, bi- or multilamellar liposomes are often present and the fraction of unilamellar liposomes in a sample depends on the lipid composition as well as on the preparation technique.

The structure of the liposomes allows for encapsulation of substances in the aqueous interior of the liposome as well as incorporation of lipophilic, or amphiphilic, substances in the membrane. This has been exploited in several areas from medicine and cosmetics to food technology. The resemblance to cell membranes have made liposomes very useful as model membranes in the field of life science.

Figure 6. Schematic illustrations of a liposome (left), and the PEG-stabilized bilayer disk (right). The PEG-lipids, marked dark gray, form the rim of the disk. The PEG-chains are included in the lower right image.

In order to disperse phospholipid bilayers in the form of liposomes energy has to be added to the system. Liposomes are thus not thermodynamically stable structures. The mechanism of liposome formation can be described in terms of the interplay between the curvature energy, $E_C$, inherent in bending of a circular flat lipid bilayer and the edge tension, $E_H$, associated with the exposure of its edge to the aqueous medium (59-61). This is the same edge tension discussed in the preceding section. The energy needed to bend the circular bilayer can be described by

$$E_C = \left(2k_C \pi r^2\right)/R^2$$
where $k_C$ is the bending modulus, $r$ is the radius of the bilayer and $R$ is the curvature radius. Upon bending the perimeter of the edge is reduced and the edge tension decreases according to

$$E_H = \gamma 2\pi r \left(1 - r^2/4R^2\right)^{1/2}$$

where $\gamma$ is the effective edge tension. The total excess energy, $E_E$, for a bilayer, bent or flat, is thus

$$E_E = E_C + E_H = \left(2k_C r^2\right)/R^2 + \gamma 2\pi r \left(1 - r^2/4R^2\right)^{1/2}$$

For a closed lipid bilayer sphere, i.e. the liposome, the edge tension vanishes. As $r = 2R$ the excess energy per liposomes thus becomes

$$E_E = 8\pi k_C$$

which is independent of the size of the liposome. With regards to the excess energy, larger liposomes will be favored over small ones whereas entropy will favor smaller liposomes.

Although the liposome is not an equilibrium structure in a thermodynamic sense liposome dispersions may, depending on the lipid composition and storage conditions, be kinetically stable for several months and sometimes years. Aggregation of liposomes is governed by the balance between attractive van der Waals forces and the repulsive electrostatic, hydration and undulation forces (62). To further improve liposome stability polymers can be associated with the liposome surface. The polymers prevent aggregation due to repulsive osmotic and entropic forces that exist between opposing polymer-decorated surfaces (2).

**Bilayer disks**

Due to the unfavorable edge tension, flat lipid bilayers are short-lived structures. However stable disk structures can be formed by the addition of micelle-forming amphiphiles, such as PEG-conjugated phospholipids (63, 64). Due to their high spontaneous curvature these have limited solubility in flat bilayers. Above the solubility limit the PEG-lipids will induce structures with high curvature. This results either in the formation of cylindrical mixed micelles or bilayer disks. The latter require a component segregation (65, 66). The PEG-lipids will in this case accumulate at the edge of the flat bilayer illustrated in Figure 6. The anticipated component segregation was experimentally shown in Paper IV. The packing of bilayer-forming lipids into a mixed micelle structure is accompanied by a bending energy penalty.
whereas the component segregation is entropically unfavorable. Which scenario takes place is largely governed by the bending modulus and spontaneous curvature of the bilayer-forming lipids (63, 66). Thus, as \( k_C \) is about 10 times higher for PC-bilayers in the \( L_\beta \) phase than in the \( L_n \)-phase, addition of PEG-lipids to PC-lipids below the \( T_m \) will induce bilayer disks whereas above \( T_m \) mixed micelles are normally formed. As mentioned earlier, cholesterol has a reducing effect on the spontaneous curvature, and an increasing effect on the bending modulus of PC-bilayers. The inclusion of cholesterol thus favors the formation of bilayer disks in PC/cholesterol/PEG-lipid systems.

Due to the open structure of bilayer disks, all lipid is directly available for interaction with the surroundings. This has rendered the PEG-stabilized bilayer disk useful as a model membrane in a number of analytical applications (67-70). Further, some initial studies on the possibility of using the PEG-stabilized bilayer disk as a vehicle for peptides and membrane proteins have been carried out (69). Some characterization and interaction studies that are relevant for this potential application were carried out in Papers III, IV and V.

Dehydration of phospholipid bilayers

Lipid nano-particles dispersed in water are typically subjected to both chemical and physical degradation (71). A slow hydrolysis of the phospholipids and a tendency of the particles to aggregate and fuse over time results in decreased shelf life for the lipid-water dispersions. Since product stability is prerequisite in drug formulation, the processes described above reduces the applicability of liposomes and other lipid particles in this area. As the degenerative processes occur more slowly in solid matter there is an interest in methods for dehydration of liposomes and related lipid structures. Commonly this is done by drying a disaccharide solution in which the lipid particles are dispersed.

One purpose of the presence of the sugars is to prevent the lipid particles from coming into close contact during the drying process. The dry sugars act as a physical barrier between the lipid particles (72, 73) and if the drying is performed properly the sugars will vitrify, i.e. transform into an amorphous matrix. It is generally considered that vitrification is essential for the protective effect provided by the sugar (74, 75). In addition, the sugars can according to the water replacement theory, replace water in the interaction with the headgroups of the phospholipids (76, 77).

When bilayers are dehydrated the lateral spacing between the lipid head groups decreases which allows for stronger van der Waals interaction between the lipids (76). As a consequence, the gel-to-liquid crystalline phase transition temperature, \( T_m \), will increase upon desiccation. A well studied example is DPPC where \( T_m \) is 42 °C for the fully hydrated lipid bilayer and
105 °C for the dehydrated bilayer. However if the lipid membranes are dehydrated in the presence of mono or disaccharides the $T_m$ can instead be reduced (78) to temperatures below those of the fully hydrated lipid bilayers.

In spite of the large interest in the sugar-lipid interactions a broad understanding of the processes taking place during dehydration of liposomes has yet not been achieved. Retention studies are commonly performed (79, 80) to evaluate effects of, e.g., sugars, matrix additives and drying kinetics on the integrity of liposomes. Little information on the mechanisms behind the loss of liposome integrity is, however, available.

In Paper V we focused on structural rearrangements of liposomes and the role of osmotic stress during dehydration by means of spray- and freeze drying. Furthermore, the effects caused by drying of lipid disks was evaluated.
Experimental techniques

Cryo-transmission electron microscopy

Cryo-TEM is unique in that it allows for direct visualization of dispersed lipids in the native aqueous environment (81). By rapidly freezing the sample an amorphous ice matrix is formed which allows for non-invasive investigations keeping the perturbations of the samples at a minimum.

The specimen are prepared by applying a small volume of the sample solution onto holey polymer film supported by a copper grid. Thin sample films that span the holes are created by removing excess solution through blotting using a filter paper. Since all investigations in this work involved dispersed lipid aggregates evaporation of water during the preparation step had to be avoided. This was achieved by doing all blotting at high humidity in a climate chamber. Immediately after blotting, the copper grid is rapidly plunged into liquid ethane kept at ~100 K in order to vitrify the sample. The sample temperature is then kept at ~100 K throughout the whole examination procedure. Fast freezing kinetics is crucial to avoid crystallization of the water. Furthermore, the fast freezing kinetics of the sample make rearrangements of the lipid molecules unlikely.

The vitrified films must be thin enough, <500 nm, in order for the electron beam to penetrate the sample. The electrons are scattered by the sample molecules when they travel through the specimen and more electron dense molecules scatter more. The resolution of the micrograph is thus influenced by the differences in scattering caused by the solvent and the lipid particles as well as by sample thickness.

A note on interpreting cryo-TEM micrographs.

In this thesis all cryo-TEM images are two-dimensional representations of the samples investigated which, to the inexperienced eye, may complicate the interpretations. Figure 7 is a useful guide to how to interpret the micrographs. One should bear in mind, though, that due to the limitations in penetrating power of the electron beam, cryo-TEM investigations of structures larger than 500 nm are difficult to accomplish.

When interpreting the micrographs obtained by cryo-TEM it is also important to keep in mind some more or less common effects that are related to the preparation steps rather than reflecting true sample characteristics. As the thickness of the vitrified films can range between 500 to 10 nm, sorting ac-
cording to size may occur, see Figure 7. Larger objects are expected to be found in the vicinity to the polymer film where the vitrified films are thickest whereas only small or flat objects can fit in the thinnest parts of the films. Invagination of liposomes may occur as a result of the osmotic gradients created during blotting. These can be wrongly interpreted as double-walled liposomes. The use of a high humidity chamber can in some cases prevent the invagination processes.

![Figure 7](image)

**Figure 7.** The illustration to the left shows how a vitrified sample of different lipid structures will appear in a 2-D image. The micrograph at the bottom shows liposomes and disks with various orientations. The illustration to the right shows the holey polymer film with vitrified sample films spanning the holes. Size sorting due to variation in the sample film thickness is also illustrated.

**Fluorescence based techniques**

**Melittin association with lipid aggregates**

A convenient way of estimating the propensity for melittin to bind to lipid aggregates is to follow the change in the intrinsic fluorescence of the peptide upon association with the aggregates. The lower polarity in the lipid environment
leads to a hypsochrome or blue shift of the emission spectrum for the tryptophan residue of melittin. This effect was used to obtain isotherms describing the association behavior of melittin with lipid aggregates in papers I-IV.

In short, melittin solutions were titrated with aliquots of the lipid dispersions and the collective emission spectrum of the melittin in the sample was collected after each addition. As more peptides associate with the lipid aggregates the blue shift increases and the shift reaches its maximum when all peptides are associated with the lipid aggregates. The shift is quantified by taking the ratio of the fluorescence intensities at 355 and 325 nm. The acquired spectra are then used to construct association isotherms. The fraction of peptide that had associated with the lipid aggregates, $\alpha$, was determined from the extent of the shift and was followed as a function of the peptide to lipid mixing ratio, $R_i$. Isotherms describing the partitioning behavior of the peptides were constructed from the fraction of associated peptide and the mixing ratios. In Papers I-III the isotherms were fitted to the expression (82):

$$\frac{(\alpha \times R_i)}{((1 - \alpha) \times [P]_{TOT})} = \frac{R_{eff}}{[P]_{aq}} = K_P \gamma_P^{lip}$$

where $[P]_{TOT}$ is the total peptide concentration in the sample, $R_{eff}$ is the actual or effective peptide to lipid ratio in the membrane, $[P]_{aq}$ is the peptide concentration in the aqueous phase, $K_P$ is the partition coefficient and $\gamma_P^{lip}$ is an activity coefficient introduced to account for deviation from ideal partitioning. In Paper II the expression used for the activity was derived from a Gouy-Chapman approach (83) accounting for mainly electrostatic interactions between the peptides whereas in Paper I and III a simpler expression was used. The latter approach accounts for electrostatic interactions as well micromechanical properties of the membranes.

One downside to employing spectroscopic techniques for studying the association of peptides to liposomes is that scattering from the liposomes may obscure the measurements. In fluorescence measurements the scattering, also known as the inner filter effect, can be corrected for by using different approaches (84, 85). In our studies the light scattered as a function of lipid concentration was estimated by optic density measurements. Those data were then used to correct the recorded fluorescence spectra.

Release of liposome content

Liposome leakage investigations were done to determine the ability of the peptides to permeabilize lipid bilayers. Briefly, a 100 mM solution of the fluorescent dye carboxyfluorescein, (CF), was encapsulated into liposomes. At 100 mM concentration the dye is 95% self-quenching. After removal of the untrapped dye the liposomes were diluted to a lipid concentration of 12 $\mu$M and mixed with the desired concentrations of the peptides. Upon mem-
brane permeabilization the liposome trapped CF is released and diluted into the external solution. As a result of the dilution CF is no longer self-quenching and the fluorescence signal increases. Thus the leakage of the liposomes is directly related to the lytic power of the investigated peptides. Leakage measurements were carried out in Papers I -III.

**Light scattering techniques**

Light that hits a colloidal solution will interact with the particles in the solution. A portion of that light will be scattered, i.e. deflected from the original direction, by the particles in a way that is dependent on the shape, size and number of particles. Large particles will scatter more as the scattered light is proportional to the sixth power of the radius\(^2\) (86, 87).

**Turbidity measurements**

The size dependency of the scattered light can be utilized to investigate structural changes in a sample, e.g. aggregation, fusion or solubilization of liposomes. The former two will lead to an increase of the amount of scattered light whereas the latter results in a decrease in scattered light. These changes can be monitored by measuring the apparent absorbance or turbidity of the sample. Turbidity measurements were performed in Paper I and III.

**Dynamic light scattering**

Dynamic light scattering; DLS, was used in paper IV and V to obtain data on the size or size distributions of the samples. In DLS the temporal intensity fluctuations of the scattered light is measured. The intensity fluctuation caused by the constant random motion of the particles is related to the translational diffusion coefficient, \(D\), of the particles. Over a period of time the scattered intensity, \(I_s\), will fluctuate around an average value. At small time intervals, \(t_0 + \Delta t\), the locations of the particles will not have changed much and the scattered intensities at time \(t_0\) and \(t_0 + \Delta t\) are highly correlated to each other. As the time intervals grow larger the correlation between the position of the particle at \(t_0\) and \(t_0 + \Delta t\) is eventually lost. The rate at which the correlation is lost is determined by the translational motion of the particles and can be described by an autocorrelation function, \(g(t)\). For a monodisperse size distribution of spherical particles the translational diffusion coefficient, \(D\), is related to \(g(t)\) according to:

\[
g(t) = \exp(-t/\tau) = \exp(-Dq^2t)
\]

\(^2\) For liposomes the scattering is proportional to the fourth power of the radius.
where $\tau$ is the relaxation time and $q$ is the scattering vector (87). For a colloidal dispersion with a distribution of sizes the autocorrelation function is determined collectively by the diffusion of all the different particle sizes. Laplace transformation or non-linear fitting procedures of the autocorrelation function allows for determination of the distribution of the diffusion coefficients. The size of a spherical particle can be deduced from

$$R_h = \frac{k_B T}{6\pi \eta D}$$

where $R_h$ is the hydrodynamic radius, $k_B$ is the Bolzmann constant, $T$ is the temperature, and $\eta$ is the viscosity of the sample.

**Small angle neutron scattering**

In Paper IV small angle neutron scattering, SANS, was employed in order to provide information on the component segregation in the bilayer disks.

Neutron scattering occurs following the interaction of neutrons with the nuclei of atoms. A unique feature of neutron scattering is that different isotopes of the same element will scatter neutrons differently. The amplitude of the scattering is determined by the scattering length of a nucleus or the scattering length density of a molecule. For instance, there is a large difference in the scattering length of the hydrogen and deuterium nuclei. Consequently water and heavy water scatter neutrons to different extents. This feature is very useful in the study of multi-component aggregates.

The intensity of the scattered beam will be dependant on the scattering angle as a consequence of the distribution of the scattering nuclei. The angular dependency is described by the magnitude of the scattering vector, discussed in the light scattering section. Furthermore, radiation scattered by different parts of a particle will be subject to interference. The extent of the interference will also be angle dependant and holds information on the shape of the particle.

In a scattering experiment the whole sample will contribute to the scattering of the incident neutron beam. Therefore, a particle must have a scattering length density that is different from that of the surrounding medium in order to be “visible”. The contrast between particle and medium grows with the difference in scattering length density. By selectively deuterating different aggregate components and matching/mismatching their scattering length densities with that of the solvent, different parts of the aggregate can be visualized (86, 88).
Spray- and freeze-drying

Spray and freeze-drying was carried out in Paper V to obtain dry formulations of liposomes and bilayer disks. Freeze-drying is the most commonly used method for dehydrating liposomes. Due to product quality and process advantages spray-drying is, however, also attracting some interest.

Dehydration by freeze-drying is achieved by cooling an aqueous solution/dispersion until ice crystals are formed (89). Dissolved and dispersed components will hence be concentrated in the non-crystallizing phase until it eventually transforms into a solid, which is often amorphous but still contains some water. The ice crystals are removed by sublimation, achieved by a reduction in pressure (primary drying). Thereafter, the remaining water is eliminated by gradually raising the temperature (secondary drying). The final product is a highly porous solid cake.

In spray-drying dehydration of the water solution/dispersion is achieved by atomizing the liquid into small droplets in the presence of a warm stream of air. The large surface area of the droplets and the heat from the drying air promotes a very rapid evaporation of the solvent (90). Dehydration of the droplets results in the formation of small solid particles. In contrast to freeze-drying the final product is a powder.

Two aspects of the spray- and freeze-drying processes that are of importance when drying liposomes and related structures are the temperature and the created surface area. Temperature changes during the dehydration process can for example impose phase transitions and structural transformations of the lipid bilayers (76, 91). The large air-liquid and ice-liquid surface areas that are created in spray- and freeze-drying respectively can affect the distribution of surface active components in the solutions/dispersion (92). Slow diffusing components, such as liposomes and related structures, may be enriched in the surface region during the spray drying process due to the rapid inwards movement of the droplet surface during the drying (93).

The role of the ice-liquid interface is not clear but it has been shown that proteins to some degree concentrate in the interfacial regions during freeze-drying (94).
Results and discussion

Peptide- lipid interactions

Melittin association to phospholipid bilayers

The toxin melittin has been studied extensively as a model peptide due to its antimicrobial properties. The peptide consists of 26 amino acid residues that adopts a more or less linear amphipathic \( \alpha \)-helical conformation at bilayers interfaces. Residues 1 -20 are mainly hydrophobic whereas the six residues in the C-terminal are hydrophilic. Four of the six positive charges are located in the hydrophilic C-terminal region (54).

The tendency of melittin to associate with lipid bilayers of different compositions was evaluated by two methods. In Paper I – IV we used the fluorescence based method, FBM, described in the Experimental techniques section. In Paper I and II melittin association was also assessed by ellipsometry. Disregarding other technical dissimilarities between the two methods, one important experimental difference is that liposomes are used as the lipid substrate in FBM whereas supported bilayers are used in ellipsometry.

One important aim for recording the association isotherms was to be able to correlate data on the membrane perturbing effects of melittin obtained under different experimental conditions, i.e., different lipid concentrations. Since all the experiments were done on liposomes the isotherms obtained by FBM was exploited for the data correlation. The association isotherms can, in addition, also reveal information on how the physical state of the lipid bilayers influence melittin association. The isotherms presented in Figure 8 reveal some clear trends that can be attributed to the cholesterol content, lipid charge and possibly the type of phospholipid.
The zwitterionic PC-systems

Addition of cholesterol decreased the affinity of melittin to the liposomes compared to the corresponding cholesterol-free systems. This is in line with earlier studies (33, 35, 95) and is a result of the condensing effect of cholesterol on phospholipid membranes. The tighter packing of the lipids, expressed as an increase in area compressibility modulus, $K_A$, will increase the cost of creating a cavity in the lipid bilayer. As bilayer penetration is part of the association process for amphiphilic peptides, a high $K_A$ hampers the transfer of melittin from the aqueous phase.

As shown in Figure 8a the isotherms suggest that melittin more readily associates with DOPC liposomes compared to the POPC liposomes. In the case of the cholesterol supplemented liposomes it is plausible that the extra double bond in DOPC makes the packing of the acyl chains less tight and thereby facilitates bilayer penetration compared to the POPC/cholesterol bilayers (20, 96). The stronger affinity of melittin for pure DOPC than for pure POPC bilayers has also been reported by others (97). It can not be explained by differences in area compressibility as $K_A$ is probably higher for DOPC bilayers (10). Circular dichroism measurements made in Paper I showed that the helical-content of melittin was higher when the peptide was associated to DOPC compared to POPC liposomes. Helix induction is thought to be an important part of the driving force for membrane association of small peptides and may to some extent account for the higher affinity...
of melittin for the DOPC liposomes. It is, however, not straightforward to see why helix formation should be promoted in the DOPC case. Perhaps the explanation for the more avid binding of melittin to DOPC compared to POPC lies in the fact that the spontaneous curvature of DOPC is slightly negative. When confined in a bilayer structure DOPC likely exposes a larger hydrophobic area as compared to POPC. The lipid cross section area for DOPC and POPC have been estimated to 74 and 68 Å² respectively (34).

![Figure 9](image)

**Figure 9.** Ellipsometry data showing the equilibrium amount of melittin adsorbed to supported bilayers. The bilayer composition was (a) DOPC, POPC, DOPC/cholesterol (60:40 mol:mol) and POPC/cholesterol (60:40 mol:mol) and the melittin concentration was 0.025, 0.1 and 0.4 μM, (b) DOPC/cholesterol (60:40 mol:mol), DOPC/cholesterol/DOPA (30:40:30 mol:mol:mol) and DOPC/cholesterol/SoyPI (30:40:30 mol:mol:mol) and the melittin concentration was 0.025, 0.1, 0.4 and 1.6 μM.

Interestingly, our ellipsometry data collected in systems based on supported lipid bilayers suggest a somewhat different trend than that exposed by the fluorescence-based studies in the liposomes systems.

The association data obtained by ellipsometry is presented in Figure 9a. Cholesterol clearly reduces the tendency for melittin to adsorb to the lipid bilayers in line with the fluorescence data. However, the results indicate that melittin adsorbs more avidly to the POPC bilayers as compared to the DOPC bilayers. Further experiments are needed to explain the discrepancy between the data obtained by the two methods. It may be related to the underlying substrate affecting the POPC and DOPC membranes differently in terms of their sensitivity to melittin binding.
Effect of negative charge on the melittin lipid interaction

Figure 8b shows the effect of supplementing POPC bilayers with 10 mol% of the negatively charged POPG. The amount of membrane associated melittin, $R_{eff}$, increases considerably. In respect of the high positive charge of the melittin molecule this effect is not surprising nor is it a novel finding (33, 98, 99). Nonetheless, it is worthy of note that the relative order of the isotherms for the PG supplemented liposome systems remain unchanged.

Figure 9b show the ellipsometry data for DOPC/cholesterol bilayers as well as DOPC/cholesterol bilayers supplemented with 30 mol% of either DOPA or SoyPI. Also in these systems the effect of the electrostatic interaction is pronounced. In fact the interaction between melittin and DOPC/cholesterol/DOPA was so strong that the fluorescence base measurements indicated that the peptide/lipid mixing ratio was equivalent to the membrane bound peptide/lipid ratio at all times.

Evaluation of the isotherms

The expression for the partitioning process presented in the Experimental techniques section and discussed in Paper II was fitted to the isotherms. The fitting parameters $K_P$, representing the partitioning coefficient, and $w$, a measure of the deviation from ideal partitioning, are shown in Table 2. As indicated above the aim was not to perform a thorough thermodynamic evaluation of the association process. It is however possible to separate the effects of bilayer charge from the chemical part of the peptide/lipid interaction, i.e. hydrophobic interactions and helix formation, as shown for several peptides including melittin (98, 100, 101). Furthermore, the contribution of $\alpha$-helix formation to the partitioning energetics for melittin and other related peptides have been addressed in number of studies, for instance see (31, 32). In our case $K_P$ should be looked upon as an estimate of the partitioning coefficient. For the charged liposomes electrostatic interactions are included in the $K_P$ reported in Table 2. Together with the fitting parameter, $w$, $K_P$ describes the association process well enough to be used for our purposes.

Table 2. Fitting parameters describing the association of melittin with liposomes. $K_P$ is the partitioning coefficient and $w$ the fitting parameter accounting for deviations from ideal partitioning. *PG and PI are negatively charged lipids.

<table>
<thead>
<tr>
<th>Lipid bilayer composition</th>
<th>$K_P$ (10$^3$ x M$^{-1}$)</th>
<th>$w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>21</td>
<td>72</td>
</tr>
<tr>
<td>POPC/cholesterol</td>
<td>28</td>
<td>5860</td>
</tr>
<tr>
<td>DOPC</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>DOPC/cholesterol</td>
<td>26</td>
<td>292</td>
</tr>
<tr>
<td>EggPC/cholesterol</td>
<td>20</td>
<td>2411</td>
</tr>
<tr>
<td>POPC/POPG *</td>
<td>2500</td>
<td>79</td>
</tr>
<tr>
<td>POPC/cholesterol/POPG *</td>
<td>56</td>
<td>421</td>
</tr>
<tr>
<td>DOPC/cholesterol/SoyPI *</td>
<td>98</td>
<td>10</td>
</tr>
</tbody>
</table>
Melittin induced membrane perturbations

1. Melittin induced leakage

The disintegrating effect of peptides on lipid bilayers is easily probed by leakage experiments in which a fluorescent dye is trapped inside liposomes. As a response to peptide-induced liposome permeabilization the dye leaks out to an extent that can be related to the amount of peptide added. Table 3 shows the melittin to lipid mixing ratios, $R_i$, and the corresponding $R_{\text{eff}}$ values, i.e. the ratio of mol membrane-associated melittin to mol lipid, needed to induce total leakage from liposomes of varying composition. For the DOPC/cholesterol a maximum of 70 -80% leakage was reached.

The data presented in Table 3 show a few interesting features. Focusing on the zwitterionic liposomes, it can be seen that DOPC liposomes have a greater ability than POPC liposomes to resist melittin-induced leakage no matter whether $R_i$ or $R_{\text{eff}}$ are considered. This confirms results reported by Rex and Schwarz (97). Perhaps slightly surprising, the addition of cholesterol to the neutral liposomes does not protect against the effects of melittin with regards to $R_{\text{eff}}$. If only the mixing ratio, $R_i$, is considered our data are in line with the common notion that cholesterol protects against melittin induced permeabilization (33, 35, 42).

The results for the negatively charged liposomes show that addition of anionic lipids offers resistance to the lytic effects of melittin in accordance with earlier studies (99, 102). The decreased ability of melittin to lyse negatively charged membranes have been attributed to a restricted mobility of the peptide as a consequence of the electrostatic interaction with the negatively charged lipids (102). Results presented in Paper II indicate that the penetration depth of melittin’s tryptophan residue, as probed by quenching measurements, decreases when the membrane is negatively charged. Melittin assumed a more shallow location in the anionic soyPI or DOPA-supplemented DOPC/cholesterol liposomes compared to neat DOPC/cholesterol liposomes. The correlation between leakage and the penetration depth of the peptide is, however, not straight forward as indicated by other studies (30, 99).

Importantly, a comparison of the leakage data for POPC/POPG and POPC/cholesterol/POPG confirms that cholesterol does not confer resistance to melittin induced leakage in terms of $R_{\text{eff}}$. 
Table 3. Leakage data. $R_i$ is the peptide to lipid mixing ratios needed to cause total leakage of CF from liposomes within 15 minutes after mixing. $R_{eff}$ is the corresponding amount of membrane associated peptide.*PA, PG and PI are negatively charged lipids.

<table>
<thead>
<tr>
<th>Lipid bilayer composition</th>
<th>$R_i$</th>
<th>$R_{eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>$8.3 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>POPC/cholesterol (60:40 mol:mol)</td>
<td>$4.2 \times 10^{-2}$</td>
<td>$5.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>DOPC</td>
<td>$2.5 \times 10^{-2}$</td>
<td>$5.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>DOPC/cholesterol † (60:40 mol:mol)</td>
<td>$7.5 \times 10^{-2}$</td>
<td>$5.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>POPC/POPG* ‡</td>
<td>$1.5 \times 10^{-2}$</td>
<td>$1.4 \times 10^{-2}$</td>
</tr>
<tr>
<td>POPC/cholesterol/POPG* ‡</td>
<td>$1.6 \times 10^{-1}$</td>
<td>$6.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>(50:40:10 mol:mol:mol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPC/cholesterol/SoyPI* ‡</td>
<td>$1.1 \times 10^{-2}$</td>
<td>$6.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>(30:40:30 mol:mol:mol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPC/cholesterol/DOPA* ‡</td>
<td>$1.8 \times 10^{-2}$</td>
<td>$1.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>(30:40:30 mol:mol:mol)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Leakage data taken from † Paper I, ‡ Paper II and ‡ Paper III. † A maximum leakage of 70-80% was registered for the DOPC/cholesterol liposomes.

2. Melittin-induced large-scale perturbations

Melittin is known to induce large-scale structural rearrangements of bilayer membranes at high peptide to lipid mixing ratios (103-108). In Papers I – II (III) structural investigations of the bilayers were done at melittin concentrations in the liposome bilayers above and below the $R_{eff}$ needed to cause maximum leakage from the liposomes. Again, the view that cholesterol acts as a protectant against melittin-induced perturbations must be modified.

Figure 10 shows DOPC liposomes exposed to different melittin concentrations. Structural rearrangements were not detected until the melittin concentration in the membranes was higher than the $R_{eff}$ that induced total leakage of the liposomes. At higher $R_{eff}$ the liposomes start to break up and open bilayers are formed. Interesting drop-shaped liposomes that have a small opening at the neck of the drop form, Figure 10c. The rupture behaviour was similar in the POPC system. The untreated POPC liposomes (not shown) were identical to the DOPC liposomes shown in Figure 10a. Open bilayer structures did not appear until $R_{eff} > 1.5 \times 10^{-3}$, i.e., after complete leakage of liposome content. Higher amounts of melittin resulted in the formation of small bilayer fragments that were stable with time, see Figure 11c.
Figure 10. Lipid structures found in the DOPC system before the addition of melittin (a), liposomes treated with melittin corresponding to $R_{\text{eff}} = 5.0 \times 10^{-3}$ (b), and $R_{\text{eff}} = 2.7 \times 10^{-2}$ (c). Arrowheads indicate open structures. Bar = 100 nm.

Figure 11. Lipid structures found in the POPC system at high melittin concentration corresponding to $R_{\text{eff}} = 7.2 \times 10^{-3}$ (a) and $R_{\text{eff}} = 2.7 \times 10^{-2}$ (b). (c) Structures found in sample (b) after 72 hours. Arrowheads and arrows indicate bilayer fragments/open structures oriented face on and edge on respectively. In (c) an ice-crystal deposited on the vitrified film is visible. Bar = 100 nm.

Examples of melittin action on cholesterol-supplemented DOPC and POPC liposomes are shown in Figures 12 and 13. In these systems we found structures indicating that melittin induces morphological changes at $R_{\text{eff}}$s below what is needed to cause total or maximum leakage. In addition to open bilayer structures large liposomes and large liposome clusters were detected. The aggregation and/or fusion processes were supported by turbidity measurements, see below. At high concentrations of melittin the bilayer structures tended to break up completely, Figures 12c and 13c.
Figure 12. Aggregate structures found in the DOPC/cholesterol (60:40 mol:mol) system before the addition of melittin (a), liposomes treated with melittin corresponding to $R_{\text{eff}} = 8.8 \times 10^{-4}$, i.e. below total leakage, (b), and $R_{\text{eff}} = 1.2 \times 10^{-2}$ (c). The inset in (c) shows the same sample after 24h. Arrowheads indicate open structures and the arrows indicate small bilayer fragments. Bar = 100 nm.

Figure 13. Aggregate structures found in the POPC/cholesterol (60:40 mol:mol) system before the addition of melittin (a), liposomes treated with melittin corresponding to $R_{\text{eff}} = 5.3 \times 10^{-3}$, i.e. just below total leakage, (b) and $R_{\text{eff}} = 1.1 \times 10^{-3}$ (c). The inset in (c) shows the same sample after 24 h. Black arrows indicate open structures. Bar = 100 nm.

To supplement the qualitative data from the cryo-TEM investigations turbidity measurements were done on the melittin-liposomes systems. They suggest that melittin induces large-scale structural changes in the cholesterol-supplemented systems already at low $R_{\text{eff}}$s, see Figure 14. The reduction in turbidity in the DOPC/cholesterol liposomes likely originates from the decreased aggregation seen in the cryo-TEM investigations, compare Figures 8b and c. The drop in light scattering was also seen in the POPC/cholesterol sample upon reexamination of the sample after 24 hours. In that case the reduced turbidity was explained by the formation of micelle-like structures.
Figure 14. Turbidity plotted as a function of peptide to lipid mixing ratio (a) and as a function of $R_{\text{eff}}$ (b). POPC (open triangles), DOPC (open circles), POPC/cholesterol (solid triangles) and DOPC/cholesterol (solid circles). The arrows indicate the $R_{\text{eff}}$ at which total leakage takes place.

For the negatively charged DOPC/cholesterol/DOPA and DOPC/cholesterol/soyPI, melittin induced liposome perturbations, that were large enough to be resolved by cryo-TEM, at $R_{\text{eff}}$S below that needed for maximum leakage, Figure 15. Compare with untreated DOPC/cholesterol liposomes, Figure 12a, for a representative image of the untreated DOPA and SoyPI-supplemented DOPC/cholesterol liposomes.

Figure 15. Aggregate structures found after the addition of melittin to (a) DOPC/cholesterol/DOPA (30:40:30 mol:mol:mol) liposomes, $R_{\text{eff}} = 1.2 \times 10^{-2}$ and (b) DOPC/cholesterol/soyPI (30:40:30 mol:mol:mol) liposomes $R_{\text{eff}} = 4.8 \times 10^{-3}$. the black arrow indicates an ice crystal. Bar = 100 nm.
3. Correlating leakage and morphological changes

Correlating the $R_{\text{eff}}$ values at which liposome rupture and maximum leakage occurs in the zwitterionic liposomes indicate an important effect of the addition of cholesterol to these systems. For the pure POPC and DOPC liposomes complete leakage was observed at $R_{\text{eff}}$s well below those needed to induce morphological changes in the systems. Yang et al showed that melittin can form toroidal pores in pure POPC bilayer (51). Although the peptide to lipid ratio used in that study corresponds to a melittin concentration in the membrane more than twice as high as that in Figure Xb. The fact that the release of liposome content was not accompanied by major morphological changes in the DOPC and POPC systems is thus in line with the notion that leakage takes place via well defined pores.

In the cholesterol supplemented POPC and DOPC systems major structural changes took place at $R_{\text{eff}}$s below those needed to induce maximum leakage. Ruptured, fused and aggregated liposomes were found to coexist with intact liposomes in these samples. As rupture inevitably leads to a complete release of the liposome content a mechanism based on a well defined pore structures can not alone account for the leakage in the cholesterol supplemented samples. Recently it was shown that whereas melittin induced leakage from DOPC membranes takes place via well defined pores, DOPC/DOPE liposomes (50:50 mol%) release their content due to a non specific process. Similar to DOPE, cholesterol tends to induce negative membrane curvature (43) and it is plausible that this effect is connected to a non specific release behavior.

In line with the results for the cholesterol supplemented DOPC and POPC membranes, melittin induced morphological changes at $R_{\text{eff}}$ values below what is needed for a complete leakage also in the DOPA or soyPI supplemented DOPC/cholesterol systems.

Alamethicin

In Paper III we investigated the interaction of alamethicin with POPC membranes containing cholesterol and/or the negatively charged lipid POPG. Alamethicin is effectively uncharged at neutral pH and should thus not discriminate between lipid bilayers with regard to their charge. Furthermore, it is suggested, albeit not undisputed, that alamethicin forms barrel-stave pores where the channels are lined with the peptide helixes without the involvement of any lipids (50, 109, 110). Whereas much research has been done to elucidate the arrangement of alamethicin in phospholipid membranes little focus has been put on alamethicin’s large-scale effects on the structure of lipid bilayers.

We investigated alamethicin’s effect on liposome structure using cryo-TEM and turbidity measurements. The presence of cholesterol in the lipo-
somes have a pronounced effect on the membrane perturbing action of alamethicin. Although alamethicin readily lyses POPC and POPC/POPG membranes, Table 4, cryo-TEM investigations and turbidity measurements revealed no large-scale perturbations at $R_i = 0.04$ (data not shown). This peptide concentration is well above the $R_i$ needed to completely leak the liposomes. Thus, permeabilization of POPC and POPC/POPG liposomes likely occurs via with the formation of small pore structures.

Table 4. The alamethicin to lipid mixing ratios, $R_i$, to release more than 90% of liposome encapsulated CF.

<table>
<thead>
<tr>
<th>Lipid bilayer composition</th>
<th>$R_i$</th>
<th>Leakage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>0.012</td>
<td>95</td>
</tr>
<tr>
<td>POPC/cholesterol</td>
<td>0.70</td>
<td>100</td>
</tr>
<tr>
<td>POPC/POPG</td>
<td>0.020</td>
<td>94</td>
</tr>
<tr>
<td>POPC/cholesterol/POPG</td>
<td>0.37</td>
<td>93</td>
</tr>
</tbody>
</table>

In contrast, addition of alamethicin to cholesterol-supplemented liposomes induces, at the same $R_i$ of 0.04, membrane rupture and the formation of rugby ball-like structures, see Figure 16. As evident from the cryo-TEM image in Figure 16b, and the turbidity measurements, shown in Figure 17, alamethicin induces fusion and aggregation in the absence of the negatively charged POPG. The ruptures seen in the large liposomes and the openings detected in the nodes of the rugby ball-like structures indicate that alamethicin, similar to melittin, can induce and stabilize open bilayer structures.

![Figure 16](image.png)

*Figure 16. Representative images of liposomes prior and after the addition of alamethicin. POPC/cholesterol/POPG in the absence of alamethicin (a). POPC/cholesterol (60:40 mol:mol) liposomes (b) and POPC/cholesterol/POPG (50:40:10 mol:mol:mol) liposomes (c) after the addition of alamethicin. Peptide to lipid mixing ratio, $R_i = 4.0 \times 10^{-2}$. Black arrowheads indicates openings or ruptures. Bar = 100 nm.*
Figure 17. Turbidity plotted as a function of time after the addition of alamethicin to POPC/cholesterol (60:40 mol:mol) liposomes and POPC/cholesterol/POPG (50:40:10 mol:mol:mol) liposomes. Alamethicin to POPC/cholesterol mixing ratio = 0.02 (open squares) and 0.04 (solid squares). Alamethicin to POPC/cholesterol/POPG mixing ratio = 0.04 (solid circles).

In line with our findings for melittin, the amount of alamethicin associated with the liposomes is expected to be lower in the cholesterol-supplemented systems than in the cholesterol-free systems, at the same $R_i$. Moreover, a more than 18 times higher $R_i$ is needed to achieve the same leakage from the cholesterol-containing liposomes as from the cholesterol free POPC and POPC/POPG systems, see Table 4. Taken together these data suggest that cholesterol modifies the mechanism for alamethicin induced liposome perturbations. However, to learn more about the impact of cholesterol on the alamethicin-lipid bilayer interactions the actual amount of alamethicin residing in the liposomes has to be determined.

Bilayer disks as model membranes and as a means to formulate and deliver peptides

The structure of the PEG-stabilized bilayer disk

The structure of bilayer disks, see Figure 6 in the Introduction, has been determined from data obtained from cryo-TEM and DLS-based studies. Due to the high spontaneous curvature of PEG-lipids they have limited solubility in bilayers. For instance, the solubility of DSPE-PEG$_{5000}$ in DSPC bilayers is ~5 mol% (64, 111). Above this concentration disk-like structures are formed. Although experimental data and theoretical calculations strongly suggest that PEG-lipids should predominantly reside on the rim of the bilayer disk, no direct evidence for component segregation can be obtained from cryo-TEM and DLS.
The component segregation was experimentally proven using SANS in Paper IV. As discussed in the Introduction, disk formation is expected to occur when the components comprising the flat part have a high net bending rigidity, $k_C$. The two compositions investigated by SANS were DSPC/DSPE-PEG$_{5000}$ (80:20) and DSPC/cholesterol/DSPE-PEG$_{5000}$ (35:40:25). The DSPC was selectively deuterated. Figure 18 displays the scattering data for neutrons in the DSPC/DSPE-PEG$_{5000}$ system at different background contrasts.

Figure 18. Neutron scattering data recorded for 5 mM DSPC/DSPE-PEG$_{5000}$ (80:20 mol:mol) in 0% D$_2$O (triangles), 50% D$_2$O (circles), 75% D$_2$O (squares) and 95% D$_2$O (diamonds). The scattered intensity, $I$, is plotted as a function of the momentum transfer. The image to the right shows a micrograph of the same DSPC/DSPE-PEG disks. Bar = 100 nm.

The data clearly indicates that component segregation takes place as it was not possible to match the scattering length density of the H$_2$O/D$_2$O background to that of the aggregates. Zero scattering would have occurred at some point for a uniform composition. Furthermore, as shown in Figure 19 a disk model could be fitted to the data obtained at 75% D$_2$O. A cryo-TEM micrograph of the DSPC/DSPE-PEG$_{5000}$ sample investigated by SANS is shown in Figure 18. The more biologically relevant DSPC/cholesterol/DSPE-PEG$_{5000}$ disks were also investigated by SANS. The data returned were in line with those obtained for the gel-phase disks.
Figure 19. Scattering data recorded for 5 mM of DSPC/DSPE-PEG$_{5000}$ (80:20 mol:mol) in 75% D$_2$O. The solid line represents the fit of a disk model to the obtained data. The model was based on a disk with a core radius of 70 Å, a corona radius of 174 Å and a height of 50 Å. The latter corresponds to the height of a DSPC bilayer in the gel phase.

Component segregation was also verified for disks where the negatively charged DPSE-PEG$_{5000}$ had been exchanged for the neutral ceramide-PEG$_{5000}$.

Peptide interactions with bilayer disks

In Paper III and IV we investigated the effect of aggregate curvature on the melittin lipid interaction. As discussed above, the results from the liposome studies showed that melittin induce formation and stabilization of open bilayer structures. Similar to the disk edge the edges of the melittin induced open bilayer structure and membrane pores are characterized by their high positive curvature. Melittin is also known to interact with micellar structures (112-114). The conformation of micellar associated melittin is similar to that of liposome adsorbed melittin, i.e., the peptide long axis is oriented parallel to the lipid-water interface (112, 113). The curvature duality of the disk structure, i.e. a flat bilayer part and a highly curved edge, encouraged us to do a comparative investigation of the association of melittin to liposomes, disks and micelles.

The liposomes and disks were composed of POPC and cholesterol and contained different amounts of ceramide-PEG$_{5000}$. At low PEG-lipid concentrations only liposomes are formed. Above the bilayer solubility limit for the PEG-lipid the fraction of disks increases and the disks become smaller as the amount of PEG-lipid is increased. At 25 mol% almost only small disks exist.
The total edge-surface area increases with the amount of PEG-lipid. To put it in another way, the aggregate mean curvature increase with increasing amount of PEG-lipid. Figure 20 shows micrographs of POPC/cholesterol/ceramide-PEG\_5000 systems. The micellar systems were lysoPC, DSPE-PEG\_5000 and ceramide-PEG\_5000.

![Micrographs of POPC/cholesterol/ceramide-PEG5000 systems.](image)

**Figure 20.** Association isotherms for melittin at 25°C in systems containing a) POPC/cholesterol/ceramide-PEG\_5000 (59.75:40:0.25 mol:mol:mol) liposomes (circles), POPC/cholesterol/ceramide-PEG\_5000 (45:40:15 mol:mol:mol) disks (squares) and POPC/cholesterol/ceramide-PEG\_5000 (35:40:25 mol:mol:mol) disks (triangles) and b) POPC/cholesterol/ceramide-PEG\_5000 (35:40:25 mol:mol:mol) disks (triangles), lysoPC micelles (diamonds), ceramide-PEG5000 micelles (circles), DSPE-PEG\_5000 micelles (squares). \( R_{\text{eff}} \) corresponds to mol associated melittin/mol lipid, and \([\text{melittin}]_{\text{free}}\) is the concentration of unassociated melittin in the aqueous phase. Displayed to the right are micrographs showing, from bottom to top, POPC/cholesterol/ceramide-PEG\_5000 (59.75:40:0.25 mol:mol:mol) liposomes, POPC/cholesterol/ceramide-PEG\_5000 (45:40:15 mol:mol:mol) disks and POPC/cholesterol/ceramide-PEG\_5000 (35:40:25 mol:mol:mol) disks. Bar = 100 nm.

The isotherms presented in Figure 20 clearly show that more melittin is associated with the particles when the amount of PEG-lipid increases. As expected, the registered \( R_{\text{eff}} \) is highest for the negatively charged DSPE-
PEG5000 micelles. Melittin binds, however, avidly also to the neutral ceramide-PEG5000 micelles. Importantly, the high affinity of melittin to the lysoPC micelles indicate that it is the highly curved surface rather than the PEG-part of the lipid that is of importance for the melittin adsorption. One reason for the impact of the curvature on melittin binding may be that more hydrophobic surface is exposed in micelles and at the rim of the disks. It is likely that the head groups in the curved structures are less tightly packed than the lipid head groups in the liposomes or the flat part of the disk. Melittin association to the curved surfaces will thus be energetically beneficial for both the disk and the peptide.

After the onset of disk formation an increase in the amount of PEG-lipid results in a larger fraction of disks, a decrease in disk size and an increase in curved surface area. Interestingly, by normalising the maximum values for $R_{\text{eff}}$ in the different ceramide-PEG5000 systems to the amount of PEG-lipid present one arrives at a common $R_{\text{eff-max}}$ of 0.16. Thus, it appears that the curvature of the lipid surface is important for the association behaviour of melittin. The SANS-investigations of disks with and without adsorbed melittin in Paper IV indicate that the association of the peptide does not affect the size or shape of the disks.

The ability to load the bilayer disks with a large amount of melittin suggests that they may become useful as vehicles for the delivery of peptide drugs. To further test melittin’s preference for curved surfaces, leakage studies were performed in the presence and absence of POPC/cholesterol/ceramide-PEG5000 disks (45:30:25 mol:mol:mol). Liposomes with different bilayer properties were loaded with the dye CF. Melittin induced leakage from the liposomes in the presence of disks were compared to that in the absence of the disks. As a consequence of the presence of bilayer disks the leakage, corrected for the amount of lipid accessible in the two cases, decreased in all liposome systems. Figure 21 shows the leakage from POPC, POPC/cholesterol, POPC/POPG and POPC/cholesterol/POPG in the presence of POPC/cholesterol/ceramide-PEG5000 disks.
Figure 21. The remaining leakage from liposomes in the presence of POPC/cholesterol/ceramide-PEG\textsubscript{5000} disks. Leakage in the absence of the disks is defined as 100% leakage.

The $\alpha$-helical peptides magainin 2 and alamethicin were also included in the leakage study. Again the peptide-induced leakage decreased in the presence of the disks, see Figure 12, indicating that a high affinity for lipid structures of high curvature may be common property of $\alpha$-helical peptides. If the disks are to be used as delivery vehicles for, for instance, antimicrobial peptides it will of course be necessary for the peptides to be able to dissociate from the disks. The high negative charge of the bacterial membrane is in this context favourable as it increases the affinity of the peptides for the bacterial membranes: compare for instance the melittin association isotherm for the POPC/POPG liposomes, Figure 8b, to that of the POPC/cholesterol/ceramide-PEG\textsubscript{5000} disks, Figure 20a.

Spray- and freeze-drying of liposomes and disks

Dry formulations of lipid structures are anticipated to have a longer shelf life than aqueous suspensions of liposomes and related structures, as discussed in the Introduction section. Furthermore, the powders produced by spray drying are suitable formulations for drug delivery via the pulmonary route. In Paper V we performed freeze- and spray-drying of liposomes and bilayer disks. Lactose was used as the carrier due to its ability to form a stable amorphous matrix with high glass transition temperature, $T_g$. Specifically, we focused on the effects that dehydration had on the lipid particle structure.

Cryo-TEM and DLS was used to characterize the structural effects. The drawback with DLS is that a defined structure has to be assumed and that existing polymorphisms are not easily resolved. Our systems were seen to contain many different types of structures. The returned hydrodynamic radius, $R_h$, should therefore be regarded as an apparent $R_h$. Furthermore, the
reported size distributions are unweighted with the result that the relative amount of larger structures will be overrated. In spite of this, the DLS data constitute a useful compliment to the cryo-TEM investigations and provide quantitative data for the whole particle population.

Osmotically induced structural rearrangements of liposomes.

Dehydration of liposomes by spray and freeze drying inevitably induces an osmotic stress on the liposome structure. To shed light upon the role of the osmotic stress on the structural stability of the liposomes we created a lactose gradient over the lipid membrane by the addition of lactose to the outside of liposomes encapsulating pure water. The final lactose concentration was 10% (w/v). The exterior lactose creates an osmotic force that causes the encapsulated water to flow out of the liposomes. The efflux of water reduces the volume of the liposomes and structural rearrangements may be anticipated to occur.

Figures 22 and 24 show cryo-TEM images of the DSPC/cholesterol and DSPC/cholesterol/DSPE-PEG$_{5000}$ systems before and after the addition of the lactose. By counting the liposomes it was estimated that roughly 1/3 of the liposomes in the non-PEGylated system were bi- or multilamellar before addition of lactose. In the PEGylated sample less than 5% of the liposomes were bi- or multilamellar. Almost all spherical unilamellar structures disappeared when the lactose was added.

![Cryo-TEM images of DSPC/cholesterol (60:40 mol:mol) liposomes prepared in water; a), and after addition of 10% (w/v) to the outside of the liposomes. Black arrows point to peanut shaped liposomes, the black arrow head denotes a totally collapsed liposome and white arrows denote partly collapsed liposomes. Bar = 100 nm.](image)

In the DSPC/cholesterol system liposomes with thick walls, built up by double or multiple bilayers, was found to coexist with a small fraction of completely collapsed liposomes. The liposomes with double bilayer walls are most likely formed by invagination of unilamellar liposomes caused by
the osmotic stress. Self closure of the invagination results in a double walled liposome encapsulating the lactose solution. By the same fashion, invagination of bi-lamellar liposomes is anticipated to result in the multi-walled liposomes. See Figure 24 for a sketch on the series of rearrangement events that may take place due to the applied osmotic stress. Invagination processes occurring at two locations on the same liposomes may explain the frequently observed peanut shaped liposomes indicated in Figure 22b.

![Figure 23. Cryo-TEM images of DSPC/cholesterol/DSPE-PEG<sub>5000</sub> (57:38:5 mol:mol:mol) liposomes prepared in water; a), and after addition of 10% (w/v) to the outside of the liposomes; b. Black arrows denote partially collapsed liposomes oriented edge-on (inset in b) and face on. Arrow heads denote totally collapsed liposomes. Bar = 100 nm.](image)

In the DSPC/cholesterol/DSPE-PEG<sub>5000</sub> system the unilamellar liposomes either collapse totally or rearrange into bi-lamellar structures upon addition of the lactose, Figure 23b. As seen from the micrograph, a large fraction of the bi-lamellar structures were also partly collapsed. This is proposed to be due to a premature self closure at a time when the liposomes still contain a large aqueous volume. Again, see Figure 24 for the proposed sequence of events that result in the formation of the partly collapsed structures. The bilayer to bilayer distance in the double liposomes roughly corresponds to twice the length of a PEG-chain. Partly collapse liposomes was also found in the DSPC/cholesterol system, denoted by white arrows in figure 22b.
Figure 24. Proposed sequence of structural events taking place as water is osmotically driven out of a unilamellar liposome. As water leaves the intact liposome (a) invagination (b) or complete collapse (c) of the liposome occurs. Self closure of the invagination results in a double liposomes (e) or, if it occurs prematurely, a two-compartment liposome (d). As water continue to flow out from the liposome in (d) the aqueous compartment collapses resulting in the structure shown in (f), top = edge-on view and bottom = face-on view.

By counting the liposomes in the cryo-TEM images it was established that the liposome radii decreased by about 20% in both systems. This is supported by the DLS data obtained before and after the addition of lactose, Figure 25. The size distributions for the osmotically shocked liposomes shift to lower radii for both the PEGylated and the non-PEGylated systems.

Figure 25. Size distributions obtained by DLS before (solid line) and after (dashed line) the addition of 10% (w/v) lactose to; a) DSPC/cholesterol (60:40 mol:mol), and b) DSPC/cholesterol/DSPE-PEG<sub>5000</sub> (57:38:5 mol:mol:mol) liposomes prepared in water.
Structural rearrangements of liposomes and bilayer disks induced by dehydration/rehydration.

Liposomes composed of DSPC/cholesterol (60:40 mol:mol) or DSPC/cholesterol/DSPE-PEG\textsubscript{5000} (57:38:5 mol:mol:mol) and bilayer disks composed of DSPC/cholesterol/DSPE-PEG\textsubscript{5000} (48:32:20 mol:mol:mol) were prepared and dried in a 10% (w/v) lactose solution by means of spray and freeze drying. The spray-dried powder and the dry cake resulting from freeze-drying was evaluated by differential scanning calorimetry, DSC. The thermal behaviour of the amorphous lactose-liposome mixture was similar to that of pure lactose. The disks had an effect on the recrystallization of the amorphous lactose. Importantly, the $T_g$ of the lactose did not change upon the presence of the lipid aggregates. As the liposomes were prepared in the lactose solution they will be subject to osmotic stress only during the dehydration and rehydration process. The disks will not be subject to any osmotic stress at all as a consequence of their open structure.

Figure 26. Cryo-TEM images of DSPC/cholesterol (60:40 mol:mol) liposomes prepared in 10% (w/v) liposomes before drying; a), after spray-drying and rehydration; b), and after freeze-drying and rehydration, c). Arrow heads in a) and c) denote ice crystals deposited after sample preparation. Bar = 100 nm.

Figure 26 show the cryo-TEM images of the DSPC/cholesterol system prepared in the lactose solution before drying and after rehydration of spray- and freeze dried liposomes. As Figure 26b and c indicates both spray and freeze-drying induce the formation of bi- or multilamellar structures. The fraction of bi- or multilamellar structures increased from ~35% before drying to ~55% and ~60% for the rehydrated spray and freeze dried liposomes respectively, as judged by the cryo-TEM images. The size distributions were shifted towards lower radii for both the spray and freeze dried samples as determined by DLS, Figure 28a. The peak centered around 800 nm indicate that a small population of large structures, not detected by means of cryo-TEM, were present in the sample that had been freeze-dried. This suggest that the majority of the structural rearrangements brought about by the dehydration process likely can be assigned to individual structural rearrangements rather than recombination processes.
DSPC/cholesterol/DSPE-PEG₅₀₀₀ liposomes prepared in 10% (w/v) lactose are shown in Figure 27. DLS data for the same liposome system is shown figure 28b. The rehydrated freeze-dried sample contained a substantial fraction of double liposomes and a few multilamellar structures, Figure 27c. Similar to the non-PEGylated liposomes, DLS investigations indicate that the freeze dried PEGylated system contained a small fraction of structures of large radii. Again, these large structures were not captured on the cryo-TEM micrographs.

Spray-drying induced substantial structural rearrangements of the DSPC/cholesterol/DSPE-PEG₅₀₀₀ liposomes. Large liposomes encapsulating other liposomes and disk-like structures were frequently observed, Figure 27b. The broadening of the size distribution revealed by DLS confirmed the cryo-TEM investigations, Figure 28b. Thus, it appears that PEGylation of the liposomes do not prevent close contact and fusion of the individual liposomes. The recombination may be a result of retarded diffusion of liposomes in the droplets. Slow diffusion may hinder an even redistribution of liposomes in the shrinking droplets as water evaporates during the drying process. A relocation of the PEG-lipid cannot explain the structural rearrangements since the composition in that case would resemble that of the DSPC/cholesterol liposomes, which did not grow in size.

Figure 28. Size distributions obtained by DLS for DSPC/cholesterol (60:40 mol:mol) liposomes prepared in lactose before drying (solid line), after spray-drying (dashed line) and after freeze-drying (dotted line) a); DSPC/cholesterol/DSPE-PEG₅₀₀₀ (57:38:5 mol:mol:mol) liposomes prepared in lactose before drying (solid line), after spray-drying (dashed line) and after freeze-drying (dotted line), b), and DSPC/cholesterol/DSPE-PEG₅₀₀₀ (48:32:20 mol:mol:mol) disks in lactose before drying (solid line) after spray-drying (dashed line) and after freeze-drying (dotted line) c).
Figure 29 show DSPC/cholesterol/DSPE-PEG₅₀₀₀ bilayer disks in a 10% (w/v) lactose solution before drying and after rehydration of spray- and freeze-dried samples. In contrast to the liposomes the disks are not subject to any osmotic stress. Nevertheless, DLS data show that the bilayer disks grow in size as a result of the drying processes. A small fraction of large band-shapes structures, indicated in 30c, and objects of irregular open structures are present in both the rehydrated disk samples. The disk growth may be related to the increased concentration of the disks upon dehydration leading to a higher probability of the bilayer disks to come in direct contact with each other. However, other processes not directly related to the distance between the bilayer disks may also promote the recombination of the disks into larger structures. The aqueous solubility of PEG increases with decreasing temperature (6) which may lead to desorption of PEG-lipids during the freezing process in the freeze-drying. In spray-drying desorption may result from a relocation of the PEG-lipids as discussed above. As the edge area decreases when the size of the disks increases the loss of PEG-lipid results in disk growth. The volume of the PEG-polymers may also be affected by sugar-PEG interactions. A reduction in the PEG-polymer volume would likely affect the distribution of PEG-lipid between the flat part and the rim of the bilayer disk. A higher solubility of PEG-lipid in the flat part of the bilayer would have the same effect as a desorption of PEG-lipids from the disk structures. Importantly, even though the mean radius of the disks increases from 20 nm to 29 and 25 nm as a consequence of spray- and freeze-drying respectively the open disk structure is retained.

**Figure 29.** Cryo-TEM images of 2.5 mM DSPC/cholesterol/DSPE-PEG₅₀₀₀ (48:32:20 mol:mol:mol) disks in 10% (w/v) lactose before drying; a), 1 mM DSPC/cholesterol/DSPE-PEG₅₀₀₀ disks after spray-drying; b), and 1 mM DSPC/cholesterol/DSPE-PEG₅₀₀₀ disks after freeze-drying, c). The arrow indicates a band shaped structure. Bar = 100 nm.

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3 Estimated from measurements of the cryo-TEM images.
Implications for drug delivery.

A major concern when dehydrating drug filled liposomes is that the drug may be released during the dehydration process. Factors such as the lipid/sugar mass ratio, the molecular size of the sugar and $T_g$ of the amorphous matrix (77, 115, 116) are of importance for the retention of the drug. Precautions also have to be taken not to pass through the $T_m$ of the lipids as maximum release of encapsulated drug is expected to occur at the gel to liquid crystalline phase transition. The implications of the structural rearrangements observed in our study on the retention of encapsulated drugs can only be speculated on without performing leakage studies.

For the delivery of peptides or proteins associated to or residing in the lipid bilayer the rearrangement of predominantly unilamellar liposomes into bi- or multilamellar structures may have important consequences. The structural rearrangements may lead to a large fraction of the active substance being located beneath one or several bilayers. The open structure of the PEG-stabilized bilayer disk, prevailing also after the dehydration and rehydration process, allows on the other hand peptides and proteins formulated in the disks to be accessible also after the drying procedure.
Conclusions

Results presented in this thesis show that the interaction between α-helical peptides, such as melittin and alamethicin, and lipid bilayers is modified by cholesterol. We verified that the cationic peptide melittin associates more readily to anionic compared to zwitterionic liposomes, and that the presence of cholesterol in the liposomes reduces the peptide’s affinity for both negatively charged and neutral liposomes. Once melittin has adsorbed to the liposomes cholesterol does, however, not offer any resistance against peptide-induced leakage. Moreover, cryo-TEM investigations revealed that the leakage from cholesterol-supplemented liposomes, in contrast to that from cholesterol-free liposomes, is accompanied by large-scale perturbations of the liposome structure. Structural investigations by means of cryo-TEM showed that cholesterol-supplemented, but not cholesterol-free, liposomes tended to change their shape, rupture and fuse in the presence of the net uncharged α-helical peptide alamethicin.

A comparative study using liposomes, micelles, and bilayer disks showed that melittin’s association with lipid surfaces is promoted by positive aggregate curvature. Consequently the curved rim of the PEG-stabilized bilayer disks could be loaded with relatively high amounts of melittin. Melittin’s preference for highly curved surfaces is shared with the peptides alamethicin and magainin 2, indicating that it may be common feature of many α-helical peptides. The partial segregation of the components of the PEG-stabilized bilayer disk, which is important for maintaining the structure of the bilayer disk, was confirmed by small angle neutron scattering.

The PEG-stabilized bilayer disks have structural properties that make them interesting for the formulation of peptide and protein drugs. A study focused on morphological effects caused by spray- and freeze-drying verified that the disks retain their open and unilamellar structure during the drying and subsequent rehydration processes. In contrast, both freeze- and spray-drying induced, due to, e.g., osmotic stress, important changes in the structure of liposomes.
Populärvetenskaplig sammanfattning


Lipider är amfifila molekyler vilket betyder att de har en del som löser sig i vatten, även kallad hydrofil, och en fet del som inte löser sig i vatten, även kallad hydrofob. Fosfolipider är en vanlig typ av lipider som ingår i cellmembran. En schematisk bild av en fosfolipid visas i figur 1. Miljön i cellen, och i de flesta fall även utanför cellen, är vattenrik. I en vattenrik miljö kommer en samling av amfifila molekyler att arrangera sig så att deras hydrofoba delar inte kommer i kontakt med vattnet. Hur molekylerna packar sig beror på deras form. Har de en cylindrisk form, som fosfolipiden i figur 1, kommer de att bilda ett dubbellager där de feta delarna är riktade mot varandra och de hydrofila huvudgrupperna mot den vattenrika miljön.

Cellmembranets struktur baseras alltså på ett dubbelskikt av lipider, men även andra ämnen som proteiner utgör en stor del av membranets massa.

Cellmembranets sammansättning är komplex både sett till hur många olika lipider och proteiner som ingår i det och till att membranens komposition varierar mellan olika organismer. Högre stående organismer cellmembran innehåller förutom fosfolipider även en stor mängd av lipiden kolesterol medan cellmembranen hos lägre stående organismer, t.ex. bakterier, helt
saknar kolesterol. När man ska studera processer som påverkar eller involve-
rar cellmembran använder man sig därför ofta av förenklade modellmem-
bran. En av de vanligaste typerna av modellmembran är liposomen, se figur
2. Den kan liknas vid ihålig sfär omsluten av ett lipidmembran. En annan typ
av modellmembran är lipiddisen. Till skillnad från den slutna strukturen
hos liposomen omsluter inte disken någon volym.

Figur 2. Bilden visar en liposom (till vänster) och en lipiddisk (till höger).

Lipiddiskens kant består av lipider som har en konisk form istället för en
cylindrisk. Dessa typer av lipider vill bilda strukturer med hög kurvatur.
Liposomerna och lipiddiskarna som vi använt i denna avhandling var av en
diameter på c:a 100 respektive mellan 30-100 nanometer (1 nanometer är en
miljarddels meter).

Ett sätt för många organismer att försvara sig mot andra organismer, ex-
empelvis för att förhinder en bakterieinfektion, är att förstöra de angripande
organismernas cellmembran. Går membranet sönder kommer cellens inne-
håll att läcka ut och cellen dör. Antibakteriella peptider är exempel på ämn-
som kan göra hål i cellmembran (en peptid kan liknas vid ett litet protein).
Många högre stående organismer, inklusive människan, producerar sådana
antibakteriella peptider. Anledningen till att peptiderna inte förstör de egna
cellerna är att peptiderna, som oftast är positivt laddade, attraheras till de
negativt laddade membranen hos bakterierna. Cellernas utsida hos de högre
stående organismerna är i stort sett oladdad. I jakten på nya antibiotika har
antibakteriella peptider blivit intressanta. Mycket forskning pågår för att
bättre förstå hur de verkar och för att upptäcka nya typer av antibakteriella
peptider som är potenta men ändå relativt sett ofarliga för människan.

I min avhandling behandlar jag i delarbete 1,2 och 3 hur peptiderna melit-
tin och alamethicin påverkar lipidmembrans barriärfunktion. Jag har använt
mig av liposomer som modellmembran. Melittin, som är huvudbeståndsdel i
bigift, och alamethicin, som är ett gift som utsöndras av en svamp, är effek-
tiva antibakteriella peptider men samtidigt är de för giftiga för våra egna
celler för att kunna användas som antibiotika. Melittin som är positivt laddad
binder starkt till liposomer med negativt laddade lipider i membranet men sämre till oladdade liposomer och sämst till oladdade liposomer med kolesterol i membranet.


I delarbete 4 studerade jag lipiddisken. Lipiddisken är en ny typ av modellmembran vars struktur har karaktäriserats av vår grupp. Målet med studien var att bevisa att diskens kant verkligen utgörs av de konformade lipiderna medan den platta delen utgörs av mer eller mindre cylindriskt förmade lipider. Vi visade även att en stor mängd peptid kan bindas till diskarna utan att diskens struktur förändras. Detta resultat tillsammans med andra studier av vår grupp har visat att disken kan vara intressant att använda för att formulera läkemedel.

TACK!

Jag har haft en fantastisk tid som doktorand. Det är så många människor som har ställt upp med sin tid och kunskap under dessa år. Om du skulle leta efter hjälpsamma och kompetenta personer kan jag tipsa om gamla och nya fysikalen, Institutionen för farmaci, mikrobiologen, Institutionen för medicinska vetenskaper, biomedicinsk strålningsvetenskap, ytbiotekniken, och Institutionen för fysik och materialvetenskap.


Martin Malmsten och Adrian Rennie för att ni nästan när som helst på dygnet svarar på och redar ut mina frågor.

Adam Lovisa Anna Malin Karin och Denny – utan er, inget att disputera på.

Göran K för att din hjälp alltid finns till hands liksom ditt glada humör.

Laila och Göran S för att ni hållit koll på mig och Mats för att du kan så mycket och gärna delar med dig av det.

Maria, Emma och Anna – utan er hade jag inte fått se en blå båt gå på grund, krupit på en äng i Polen efter en vigselring eller sprungit med famnen full av champagne på Carrefour i Grenoble. Tur för mig att ni finns!

Malin och Karin – tack för att ni har stått ut med mig och hjälp till så mycket på sluttampen.

Min familj – ni är min trygghet!

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Acta Universitatis Upsaliensis

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Editor: The Dean of the Faculty of Science and Technology

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