Development and Evaluation of Lipodisks Intended for Use as Biomimetic Membranes and Drug Carriers

MALIN MORIN ZETTERBERG
Polyethylene glycol-stabilized lipodisks have emerged as a novel type of lipid-based nanoparticles with high potential as both drug carriers and biomimetic membranes. In this thesis we assess both of these applications, and show how the properties of the lipodisks can be further developed and optimized.

Initially, we show that the antimicrobial peptides melittin, alamethicin and magainin 2, in spite of their very different physico-chemical properties and suggested modes of action on membranes, all have high affinity to lipodisks. Using melittin as a model peptide, we confirm a maintained antimicrobial effect of disk-formulated peptides. We also show that melittin dissociates slowly from the disks, resulting in extended drug release and prolonged antibacterial effect. Additionally, we present evidence that the peptide is protected against enzymatic degradation when formulated in the disks.

Further, we develop a stable HPLC-MS system with immobilized lipodisks as model membranes. The stability of the system is confirmed by drug partitioning analysis using 15 different drug compounds. We also show how the lipodisk column can be supplemented with cyclooxygenase by in situ incorporation of the protein in the lipodisks. The specific binding of the protein to the disks is confirmed using QCM-D.

Finally, by changing the polymer length and applying a new preparation protocol, we have optimized the lipodisks for use as drug carriers and biomimetic membranes. Previous lipodisk studies have been conducted on systems containing PEG-lipids with polymer molecular weights of 2000 or 5000 Da. Also, conventional protocols for the preparation of lipodisks typically require a PEG-lipid concentration of 15 mol% or more. Here we show that stable lipodisks can also be produced using PEG-lipids with a 1000 Da molecular weight polymer and that the use of shorter PEG-lipids dramatically improve the amount of lipodisks that can be immobilized on silica surfaces. Moreover, through the development of a method in which lipid mixtures are sonicated at low temperatures, we produce lipodisks containing as little as 2 mol% PEG-lipid. We present data verifying that these disks are superior to disks with higher PEG-lipid content in terms of their ability to incorporate externally added PEG-lipids functionalized with targeting agents.

Keywords: model membranes, drug delivery, drug partitioning, antimicrobial peptides, nanocarriers, cryo-TEM, polymer-stabilized bilayer disks
Till Kerstin och Knut
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


(*) First authorship is shared by these authors

IV  Morin Zetterberg M., Ahlgren S., Agmo Hernández V. and Edwards K, Optimization of Lipodisk Properties by Modification of the Extent and Density of the PEG Corona. *manuscript*

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<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
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<td>CAP</td>
<td>Cationic antimicrobial peptide</td>
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<tr>
<td>ceramide-PEG&lt;sub&gt;n&lt;/sub&gt;</td>
<td>N-palmitoyl-sphingosine-1-(succinyloxy(polyethylene glycol)&lt;sub&gt;n&lt;/sub&gt;)</td>
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<td>CF</td>
<td>5(6)-carboxyfluorescein</td>
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<td>Chol</td>
<td>Cholesterol</td>
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<td>cmc</td>
<td>Critical micelle concentration</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>cryo-TEM</td>
<td>Cryo-transmission electron microscopy</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DSPC</td>
<td>1,2-distearoyl-&lt;em&gt;sn&lt;/em&gt;-glycero-3-phosphatidylcholine</td>
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<td>E. coli.</td>
<td><em>Escherichia coli</em></td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>MP-SPR</td>
<td>Multi-parametric surface plasmon resonance</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>OG</td>
<td>Octyl β-D-glucopyranoside</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-&lt;em&gt;sn&lt;/em&gt;-glycero-3-phosphatidylcholine</td>
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<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-&lt;em&gt;sn&lt;/em&gt;-glycero-3-phosphatidylglycerol</td>
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<tr>
<td>QCM-D</td>
<td>Quartz crystal microbalance with dissipation monitoring</td>
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<td>Soy PE</td>
<td>Soy L-α-phosphatidylethanolamine</td>
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<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Gel-to-liquid-crystalline phase transition temperature</td>
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<tr>
<td>UV detection</td>
<td>Ultra-violet detection</td>
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<tr>
<td>WAC</td>
<td>Weak affinity chromatography</td>
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1. Introduction

1.1 Amphiphilic molecules and self-assembly

The immiscibility of oil and water is well known and many are familiar with the eventual phase separation into two discrete phases that occurs in oil-water mixtures. The reluctance for these liquids to mix originates from their different types of intermolecular interactions. Depending on their properties, molecules are often described based on their solvent preferences as being either hydrophilic (water-loving) or hydrophobic (water-hating).

However, all molecules cannot be separated into water-loving or water-hating substances. In fact, there is a large class of compounds that have both hydrophilic and hydrophobic parts. These are known as amphiphiles, due to their ambivalent nature. Other common names for amphiphilic molecules are surfactants or detergents. A schematic illustration of an amphiphile is shown in Figure 1.

Amphiphilic molecules can be dissolved in water at low concentrations. However, as a result of their partial hydrophobicity, increasing amphiphile concentrations eventually leads to molecular aggregation and self-assembly. The monomer solubility of an amphiphilic molecule will depend on the properties of both the amphiphile and the solvent.

The driving force for self-assembly at high concentration is the large energy cost of dissolving hydrophobic compounds in water, commonly known as the hydrophobic effect (1-3). The unique solvent properties of water can largely be explained by the molecular structure of this special compound. Due to the pronounced difference in electronegativity between hydrogen and oxygen, there will be large dipole moments along the O-H bonds of the wa-
water molecules. Each molecule can therefore be described as having two positive charges centred on the hydrogen atoms and with the two unshared electron pairs represented as two negative charges on the opposite side of the oxygen atom. This tetrahedral geometry allows the water molecules to hydrogen-bond with each other and form three-dimensional network structures. In pure water the molecules will hydrogen-bond with each other in all possible directions with a large degree of configurational freedom. Solvation of a molecule that is unable to form hydrogen bonds imposes a reorganization of the surrounding water molecules. Since this organization will cost the system in free energy, there is an entropic gain to be made from aggregation of amphiphiles in structures where the hydrophobic tails are shielded from the surrounding water.

1.1.1 Structures in self-associated samples

Self-assembly in surfactant systems can result in a variety of structurally different aggregates ranging from globular micelles to far more intricate structures with surfactant-lined channels spanning throughout the aggregates. The type of aggregates formed is dependent on a large number of parameters including pH, ionic strength and total surfactant concentration. Precise predictions of the structural arrangement in self-associated samples are therefore difficult. It is, however, possible to roughly estimate the aggregate structure based on the concept of a packing parameter, $N_s$ (4). The packing parameter is represented by

$$N_s = \frac{v}{a_0 l_c}$$

where $v$ is the hydrophobic volume, $a_0$, the optimal head-group area and $l_c$ is the critical length of the hydrophobic chains. This parameter assesses the degree of conic shape of the individual molecules by giving the ratio between the actual molecular hydrophobic volume and the volume of a cylinder with the same area as the surfactant head group and length as the hydrophobic chains. A schematic illustration of how the aggregate structure varies with increasing packing parameter is shown in Figure 2. Low $N_s$ values typically lead to the formation of highly curved aggregates, like the globular micelle shown in the top left corner of Figure 2. Amphiphiles with $N_s$ close to one are cylindrical and can therefore pack in flat bilayer structures.

By convention, the aggregate curvature is defined as being positive when the polar interface is bent towards the non-polar region and negative when it is curved towards the water. Zero curvature is found in structures with planar interfaces.
Figure 2. A schematic overview showing the sequence of preferred aggregate structures as the packing parameter of the amphiphiles increases.
1.1.2 The lamellar phase and liposome formation

Phospholipids are a class of amphiphilic molecules of very high biological relevance. These molecules constitute one of the main components in the architecture of biological cell membranes and are therefore abundantly present throughout all living organisms. The bilayers formed upon the spontaneous association of phospholipids constitute efficient barriers that are largely impermeable to molecules in their surroundings. These barriers enable the controlled exchange of solutes between the cell interior and exterior and are therefore a prerequisite for life.

Phospholipid molecules consist of a glycerol backbone linking two ester-bound hydrophobic fatty acid chains to a hydrophilic head group. There is a large variability in fatty acids regarding length and degree of saturation making the phospholipids a diverse group of molecules. Additionally, a variety of phospholipid head groups exist, with phosphatidylcholine (PC) being one of the most abundantly found in nature. The molecular structures of the lipids used in this thesis are shown in Figure 3.

![Figure 3. Molecular structures of the phospholipids used in this thesis.](image-url)
Most phospholipids have a nearly cylindrical shape and in water they will therefore pack as bilayers in lamellar structures. As a result of their comparatively long hydrophobic chains, these lipids often have very low aqueous solubility, typically in the nanomolar range or lower.

Fully hydrated phospholipid bilayers can undergo a number of temperature-dependent phase transitions (2, 5). At low temperatures the bilayers are found in a lamellar crystalline phase, \( L_C \), where the molecules are densely packed. Increasing the temperature will at some point lead to transformation of the bilayer into a lamellar gel phase, \( L_{\beta} \). In this phase, the lipids still have a high degree of conformational order but they are less tightly packed than in the crystalline phase. Increasing the temperature further will eventually lead to a transformation of the gel-phase membrane into a liquid-crystalline phase, \( L_{\alpha} \). Lipids in the \( L_{\alpha} \) state are disordered and exhibit rapid lateral diffusion. The temperature at which the \( L_{\beta} \)-to-\( L_{\alpha} \) transition occurs is called the main transition temperature, \( T_m \), and it is greatly affected by the length and degree of saturation of the hydrophobic chains. Longer fatty acids, as well as a high degree of saturation, will favour the gel-phase stability and thereby lead to the phase transition taking place at higher temperatures. Additionally, some lipid classes can adopt yet another phase called the rippled phase, \( P_{\beta} \), between \( L_{\beta} \) and \( L_{\alpha} \).

Cholesterol, a small molecule found in most eukaryotic membranes, has a profound effect on the properties of phospholipid membranes. The molecular structure of cholesterol is shown in Figure 4. Like the phospholipids, it is amphiphilic, but the structure of this molecule is very different from those shown in Figure 3. The hydrophobic part consists of a large stiff hydrocarbon ring system and the hydrophilic contribution comes from a hydroxyl group attached at one end of the molecule. In phospholipid bilayers, cholesterol inserts with the hydrophilic group in close proximity to the ester bonds of the fatty acids and with the hydrophobic part in the membrane interior (6). When situated in a gel-phase bilayer, cholesterol will break the packing order of the lipids and induce disorder in the acyl chains while the opposite is true in a liquid-crystalline membrane (7, 8). Upon inclusion of 20-30 mol% cholesterol in a phospholipid bilayer, the membrane will transform into a new phase, the liquid-ordered phase, \( L_o \). Compared to the liquid-crystalline phase, liquid-ordered membranes constitute barriers of low permeability and high mechanical coherence. Lipids in liquid-ordered membranes exhibit a high degree of order while still having high diffusional rates, making these membranes both rigid and flexible.
Dilute phospholipid lamellar phases can be used to produce liposomes. Provided that the cost in bending energy is not too high, the edge tension will cause dispersed bilayer sheets to curve and self-close in order to eliminate the unfavourable edges (9). The resulting liposome has a closed bilayer structure with an aqueous interior. A schematic illustration of a liposome is shown in Figure 5. Because of their high bending rigidity, gel phase membranes do not self-close and liposomes can therefore only be prepared at temperatures above the main transition temperature of the lipids used. Since their discovery in the mid-1960s (10), liposomes have found use in a wide range of applications from medicine and cosmetics to food technology.
1.1.3 Lipodisks

As mentioned in the previous section, small bilayer fragments are short-lived structures and in systems where they are unable to self-close into liposomes they rapidly fuse into large lamellar sheets. It has been shown, however, that small bilayer fragments can be stabilized and prevented from fusion by the addition of micelle-forming molecules that accumulate at and stabilize the fragment edges (11-13).

A particular group of molecules that has been shown to very effectively stabilize these bilayer disk structures is phospholipids that have a polyethylene glycol (PEG) moiety conjugated to their head group (14-16). The large hydrophilic PEG causes a dramatic increase in the effective head group area of the lipids. Consequently, these PEG-lipids will, in accordance with the concept of packing parameter previously mentioned, be more prone to form structures of positive curvature (17). Low amounts of PEG-lipids can be included and dissolved in phospholipid membranes and PEG-lipid inclusion is a well-known strategy for increasing both the colloidal stability and in vivo circulation time of liposomes (11, 18-20). However, upon reaching membrane saturation of PEG-lipids the liposomal structure will start to break up into smaller aggregates and at very high PEG-lipid amounts the mixtures form globular mixed micelles.

In the interval between liposome and globular micelle formation, circular bilayer structures, lipodisks, can be formed. The structure of these disks is shown in Figure 6. Lipodisks are composed of a flat lipid bilayer surrounded by a highly curved rim. The disk structure is obtained as the result of partial component segregation where the PEG-lipids, due to their preference for positively curved surfaces, reside mainly at the rim.

![Figure 6. Two different schematic illustrations of PEG-stabilized lipodisks. Bilayer-forming lipids are shown in light grey and PEG-lipids in dark grey.](image-url)
The component segregation in lipodisks has been confirmed both experimentally and with molecular simulations (15, 21). Complete mixing of the lipid components within the aggregates would result in structures where all lipids are located at a positively curved surface. Such an organization would be beneficial in terms of entropy but would also impose an energetic cost from packing lipids that prefer planar structures into positively curved aggregates. The structures obtained in PEG-lipid/lipid mixtures are a compromise between these two counteracting forces, and lipodisks therefore only form when the entropic loss is small in comparison with the cost of bending the planar bilayer. This is consistent with the fact that, although they have been observed in liquid-crystalline membranes (22), lipodisks typically form in mixtures of PEG-lipids and rigid lipid bilayers in the \( L_\beta \) or \( L_o \) phased state (11, 14, 16, 22).

The onset of lipodisk formation usually occurs in the range of 5 to 10 mol\% PEG-lipid and at this PEG-lipid content, lipodisks are found in coexistence with liposomes. Increasing the PEG-lipid content will gradually reduce the amount of liposomes in the samples. Typically about 20 mol\% PEG-lipid is required in order to obtain pure lipodisk preparations. The PEG-lipids used to produce disks in this thesis are shown in Figure 7. Lipodisks show great colloidal stability, most likely as a result of the steric repulsion afforded by the polymer chains surrounding them (3, 23, 24). High thermal stability of the disks has also been shown and once disks have formed in gel-phased membranes, they remain stable upon heating the samples above the lipid transition temperature (14, 16). Additionally, as a result of the low aqueous solubility of the individual components, lipodisks show excellent stability upon dilution. The critical micelle concentrations (cmc) of the PEG-lipids shown in Figure 7 are in the low micromolar range and the monomer solubility of these lipids will be even lower in the presence of lipodisks. Lipodisks have previously been prepared using a large variety of lipids (11, 14-16, 25, 26) and the possibility of including lipids of different charges, acyl chain lengths and lipid head-groups offers a large variability in the lipodisk properties. The size of the disks varies with composition and preparation technique and lipodisks can be prepared in the size range of a few tens up to a few hundred nanometres. Size optimization can be achieved by either altering the PEG-lipid/lipid ratio or by choosing a suitable preparation method.

As will be discussed in the following sections, the open bilayer structure in combination with their excellent long-term stability and stability against dilution make lipodisks suitable for use in several different applications.
1.2 Lipodisk applications

1.2.1 Drug delivery

The design of new drug delivery systems has been of significant interest in recent decades and there are several advantages that can be foreseen from the formulation of drug molecules in carrier vehicles. Apart from the problem that many newly developed drugs have low aqueous solubility and are therefore impossible to administer in free form, formulation in carriers can also help protect therapeutic substances from enzymatic degradation and ensure controllable, sustained release of the drug. By selectively targeting specific cells or tissue, the drugs can be concentrated at their desired site of action, thus reducing systemic side effects (27-29).

Lipodisks hold great potential as carrier systems in drug administration. Since they are composed of non-toxic endogenous molecules, these nanometre-scale aggregates are ideal as carrier particles. As mentioned earlier, the lipid composition can be altered and tuned in order to optimize the disk
properties with regards to important parameters like size and surface charge. Surface grafting of polymers is a strategy that is frequently used to increase the systemic lifetime of drug carriers (30-32) and it is well known that membrane inclusion of PEG-lipids increases the stability and circulation time of intravenously administered liposomes (18, 19). Similarly, Zhang et al. have recently confirmed long in vivo survival also for lipodisks (33).

Additionally, the PEG-lipids surrounding the disks can be used to link specific targeting agents, such as ligands and antibody fragments, to the lipodisk surface. By including suitable targeting agents that bind selectively to selected receptors on the cell surface, drug delivery systems that efficiently target specific tissue or cells can be developed using lipodisks. Of the many parameters that determine the in vivo kinetics and final biodistribution of a carrier system, particle shape is one that has recently been brought to light (34, 35). Disk-shaped particles have been shown to have a number of advantageous properties and even though this parameter is not yet fully investigated, the non-spherical shape of the disks may prove beneficial for drug delivery.

The lipodisk structure offers possibilities of formulating both hydrophobic substances that can be solubilized in the lipodisk interior as well as surface-active compounds that may intercalate at the lipodisk surface. Additionally, by including charged lipids, such as the negatively charged DSPE-PEG molecules shown in Figure 7, drug loading can be achieved by electrostatic attraction. A schematic illustration summarizing the possible uses of lipodisks in drug delivery is shown in Figure 8.

![Figure 8. Schematic illustration showing the different possible uses of lipodisks as drug delivery systems.](image-url)
Liposomes are frequently used as delivery systems for both hydrophilic and hydrophobic drugs (36). However, the usefulness of liposomes as carriers of drugs that in themselves have membrane-associating properties can sometimes be limited. Since inclusion of additional molecules in a phospholipid bilayer can impose a stress in the bilayer, it can therefore also lead to membrane disruption. As an example, it has been shown that the antimicrobial peptide melittin can induce liposome rupture and fusion in liposome suspension of various lipid compositions (37, 38). It is likely that the open bilayer structure of the lipodisks makes them more resistant to such membrane-disturbing molecules and that these structures are therefore able to carry comparatively high loads of membrane-active drugs.

Melittin, mentioned above, belongs to a large class of membrane-associating molecules known as amphiphilic antimicrobial peptides (AMPs). Antimicrobial peptides are omnipresent and have an important function as part of the innate immune system in practically all living organisms (39, 40). Many of these peptides exert their antimicrobial effect by direct action on the bacterial membranes. Because of their special mode of action, AMPs have received a lot of attention as potential drugs for overcoming the ever growing problem of antibiotic resistance. Disruption of the cell membrane integrity is very efficient and it offers a comparatively un-specific mechanism for which it is likely too difficult for the bacteria to develop resistance against.

One class of AMPs that has recently received a lot of attention is the cationic antimicrobial peptides (CAPs). In addition to their antibacterial effect, CAPs have shown other promising properties, making them useful both in treating various types of cancer and for immunoregulation (41, 42).

However, in spite of several reports on positive therapeutic effects from these peptides, there has been very little success transferring these effects into clinic. One major reason that only few AMPs have emerged as drugs is the difficulty in administering these small biomolecules. Antimicrobial peptides are very rapidly degraded and show high in vivo clearance rates. One possible solution to the problems involved in AMP delivery could be to formulate these drugs in a suitable carrier. There are reasons to suggest that lipodisks hold great potential to be used as AMP carriers. In addition to the many beneficial properties possessed by these structures, melittin has been shown to bind avidly to the curved rim of lipodisks (15). This high affinity for lipodisks may very well be shared by several other AMPs and lipodisk formulation could turn out to be a promising solution for AMP delivery.
1.2.2 Model membranes

Biological membranes are essential for life. They have fundamental compartmentalization functions and constitute the barriers that control interaction between living cells and their surroundings. Apart from their barrier function, membranes control and regulate several important processes including signal transduction and enzyme activities. In addition to the plasma membranes, eukaryotic cells have membranes surrounding the nucleus and organelles – and of course also in the endoplasmic reticulum and Golgi apparatus. Because of their important functions, there is a lot of interest in studying processes that occur at these important surfaces. As mentioned earlier, phospholipid bilayers constitute the basic unit in cell membranes and they are therefore frequently employed in the construction of relevant model systems.

Molecules can enter cells either via active, protein-mediated transport or by passively diffusing through the cell membranes. For many drugs, the passive diffusion across the membrane will greatly influence the drug’s ability to reach its target. The distribution into lipid bilayers is therefore a crucial parameter that needs to be assessed when developing new drugs. Different types of phospholipid bilayers have been used as model membranes in drug partitioning studies. Previously reported systems include supported membranes (43, 44), micelles (45), liposomes (46, 47), and lipodisks (24-26, 48). Johansson et al. showed that lipodisks are superior to liposomes as model membranes in drug partitioning studies due to their open bilayer structure, which enables accurate determination of the lipid volume accessible for drug interaction (24). The possibility of including functionalized groups on the lipodisk PEG-chains allows for disk immobilization on a solid substrate through a large variety of different coupling mechanisms. Lipodisk immobilization on SPR and QCM-D surfaces using functionalized PEG-lipids has been reported previously (49, 50). Combining the beneficial properties of lipodisks as model membranes with the possibility to immobilize them on a surface could open up for the production of stable chromatographic systems of high biological relevance for the acquisition of drug/bilayer partitioning data.

Apart from controlling passive diffusion of solutes, phospholipid bilayer membranes constitute the native environments for the enormous amounts of proteins that reside in biological membranes. These proteins serve as gatekeepers and control both molecular transport and signal transduction through the membrane. Because of their important functions, a lot of interest has been focused on membrane-associated proteins and today they constitute the largest class of targets in drug discovery and development (51). However, studying membrane proteins can be a challenging task since they are sensitive to the surrounding environment and studies of them must be made using suitable membrane models (52). A large number of different membrane
models have been suggested, including different types of supported mono-
layers (53, 54) and bilayers (55, 56) as well as proteoliposomes (57) and
disk-shaped lipid aggregates (58, 59). Many of these systems do, however,
have drawbacks in terms of protein activity and stability, as well as difficul-
ties in controlling accessibility to the protein’s active site. Hence, there is
still a great need for the development of new, improved, biomimetic mem-
brane models.

One approach to improve on the problems involved with previously re-
ported techniques could be the use of immobilized lipodisks as model mem-
branes. The possibility of reconstituting proteins in the disks has been con-
firmed by Johansson et al. (26). By choosing suitable lipids, the disks can be
made to effectively mimic the native environment of the protein of interest.
Further, the open disk structure can be expected to benefit membrane-protein
interaction studies since all proteins in the disks, regardless of their orienta-
tion in the membrane, will have their active site exposed to the surrounding
media.

1.3 Aims

The aim of this thesis has been to develop and evaluate lipodisks as both
drug carriers and model membrane systems.

In Paper I, the structural reorganization and induced leakage in liposome
suspension upon addition of antimicrobial peptides was investigated. Peptide
affinity for lipodisks was assessed by determining whether the peptides bind
preferentially to the disks as compared to liposomes.

In Paper II, the lipodisks’ suitability as carriers of amphiphilic antimicro-
bial peptides was assessed. System performance was evaluated in terms of
drug-loading capacity, release profile and drug stability upon lipodisk for-
mulation.

In Paper III, we studied immobilized lipodisks as model membranes in
HPLC columns. The aim in this paper was to develop a stable system suita-
ble for high-throughput analysis. Additionally, the possibility of incorporat-
ing a membrane protein was assessed in this paper.

The objective of Paper IV was to develop improved lipodisks intended for
both drug delivery and model membrane applications and to further under-
stand the fundamental aspects of lipodisk formation. In this paper, we ex-
plore the possibility of achieving improved surface immobilization and in-
corporation of targeting agents by alterations in the polymer chain length and
amount of PEG-lipids used in lipodisk production.
2. Experimental techniques

2.1 Lipodisk preparation

Lipodisks can be produced through a variety of techniques and the choice of preparation path affects both the size and the homogeneity of the produced disks (22). The lipodisks used in this thesis were prepared through sonication (Papers I, II and IV), detergent depletion (Paper III) and extrusion (Paper IV). Common for all preparation methods is the initial production of a lipid film that is formed by co-dissolving the desired lipids and PEG-lipids in chloroform and allowing the mixture to dry in a vacuum oven.

Sonication is a high-energy input technique that can be used to produce homogenous samples of small lipodisks (22). During sonication, the lipid samples are dispersed into small lipid fragments and the lipodisk size obtained using this method is likely explained by the PEG-lipids sterically hindering fusion of these small fragments once they have been formed (60).

The second preparation method used in this thesis, detergent depletion, leads to the formation of very small disks (22). First, the lipid film is solubilized using a concentrated surfactant solution. Detergent removal is then obtained by elution of the sample on a size exclusion column. Due to its comparatively high solubility, the detergent will gradually be depleted from the sample as it distributes into the surrounding buffer. As the detergent concentration decreases the aggregates will fuse and grow in size until reaching a final state. Similarly as with sonicated lipodisks, the small aggregate size obtained with this method is likely an effect of steric repulsion between the included PEG-lipids.

In Paper IV lipid samples were prepared by initial hydration of the lipid film using buffer followed by extrusion. In the extrusion process, the samples are pushed back and forth several times through a polycarbonate filter with a 100 nm pore size. In comparison with both sonication and detergent depletion, the extrusion technique gives larger disks and the samples are typically more heterogeneous.
2.2 Cryo-TEM

Cryogenic transmission electron microscopy (cryo-TEM) is a very useful technique for studying dilute aqueous samples of phospholipid aggregates as it allows for direct visualization of sample morphologies (61-64). The method is based on ultra-rapid cooling of thin liquid films and provides specimens that can be observed close to the native state of the original sample. The rapid cooling of the sample is too fast for the water molecules to organize into ice crystals and instead amorphous solid water samples are obtained. As a result of the vitreous state of the water, electrons are able to penetrate the thin samples and aggregates within the film can therefore be visualized. The fact that the cooling is rapid enough to prevent even the small water molecules from crystallizing makes it highly unlikely that the much larger phospholipid aggregates in the samples will have time to reorganize significantly during this process. Since no fixation or staining is used in this method, the risk of artefact introduction is comparatively low resulting in very accurate sample characterization.

The sample preparation used in this thesis is conducted as follows. A small aliquot of sample is deposited on a copper grid covered with a holey polymer film followed by blotting with a filter paper to remove excess solution. This procedure results in very thin, 10-500 nm, liquid films spanning the holes of the grid. The preparation step is performed in a climate-controlled chamber ensuring constant, controllable temperatures and high relative humidity. The experimental setup for sample preparation is shown in Figure 9. Immediately after blotting, the grid is plunged into liquid ethane held at ~100 K, which causes instant vitrification of the sample. In order to maintain the vitrified state and to avoid water condensation, which can lead to ice-crystal formation on the surface, the samples are then carefully handled and kept at low temperatures in a nitrogen atmosphere upon transfer to their final position in the electron microscope. Analysis is then performed by electron irradiation at low pressure, resulting in two-dimensional representations of the aggregate structures in the films. A schematic illustration showing how different 3-D structures appear in cryo-TEM analysis is shown in Figure 10.
The particles visualized with cryo-TEM need to be in a size range of roughly 4 to 500 nm. The minimum size requirement is due to the resolution limit and the upper limit is set by the increased scattering contribution from the vitrified water in thick sample films. When drawing conclusions from cryo-TEM images, one should be aware of artefacts that can sometimes appear in this technique. One such possible artefact is aggregate size-sorting as a result of variations in the sample film thickness. The sample blotting process will result in thin liquid films spanning the holes of the polymer covering the cupper grid. These films will be shaped like concave lenses, wherefore larger aggregates are often found close to the edges of the films and smaller ones closer to the centre. Because of the size-sorting effect, it is important to investigate large specimen areas in order to ensure that no information about the aggregate structure is missed. Provided that such care is taken, cryo-TEM can be used for size determination and it has been shown that reasonable agreement in the determined aggregate size using cryo-TEM, freeze fracture EM and dynamic light scattering can be obtained (65).

Figure 9. Schematic illustration of the climate-controlled preparation chamber used to prepare samples for cryo-TEM analysis. © Göran Karlsson
Figure 10. The two pictures at the top show a schematic representation of how a vitrified sample appears in a two-dimensional projection. Shown below is a cryo-TEM micrograph in which both liposomes and lipodisks of different orientations can be observed. The scale bar in the micrograph is 100 nm.

Another artefact, which is likely of minor importance when investigating lipodisk samples, is the effect of osmotic stress during sample preparation. Evaporation of water can result in an osmotic pressure difference between the interior of liposomes and the surrounding buffer. This gradient will lead to water transport from the liposome interior followed by the formation of invaginated liposome structures. This effect can, however, be reduced by ensuring high humidity during sample preparation and changes in osmotic pressure are unlikely to have any significant effect on the structure of lipodisks.

Finally, it is important to realize that, although excellent qualitative characterizations can be made with cryo-TEM, the technique is not quantitative. In order to determine the overall structural properties of a sample correctly, cryo-TEM should therefore be used in combination with a quantitative analysis technique.
2.3 Dynamic light scattering

Dynamic light scattering (DLS), also known as quasi-elastic light scattering, is a useful technique for determining size distributions of small particles in solution. This technique probes the diffusional rate of the particles by monitoring fluctuations in the scattered light and, from that, particle size can be determined (66).

Small particles scatter incident light in all directions and the total scattering from a sample will be dependent on the relative distances between the scattering objects. Due to the random motion of the particles, the scattering intensity will therefore fluctuate and the intensity correlation will eventually be lost. Based on the time scale of this correlation decay, an auto correlation function, g(t), is determined, from which the translational diffusion coefficient, \( D \), can then be calculated.

The hydrodynamic radius, \( R_h \), is then calculated using the Stokes-Einstein equation

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

where \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature and \( \eta \) is the solvent viscosity. When using DLS for characterization of non-spherical particles, it is important to realize that the hydrodynamic radius determined with this technique is based on the assumption that all scattering objects are spherical.

DLS was used in combination with cryo-TEM to determine particle size distributions in Papers III and IV.

2.4 Leakage assays

Phospholipid membrane permeabilization was studied in Papers I and II by measuring liposome leakage of the hydrophilic fluorescent dye 5(6)-carboxyfluorescein (CF). At low concentrations of CF the fluorescence intensity is linearly dependent on the number of dye molecules present in the solution. But increasing the dye concentration will effectively reduce the fluorescence, due to molecular self-quenching, rendering the sample practically non-fluorescent at high concentrations (60, 67). The principle of the experiment is schematically shown in Figure 11.
Figure 11. Schematic illustration of the leakage assay used in Papers I and II. The left image shows a liposome encapsulating CF at a high concentration and is therefore not fluorescent. As the liposomes leak their content, the dye becomes diluted in the surrounding solution and fluorescence is detected.

By encapsulating CF in liposomes at a self-quenching concentration followed by removal of external dye, leakage can conveniently be monitored by the increase in fluorescence as the dye becomes diluted in the surrounding buffer. The external dye after liposome production was removed by changing the outside buffer on a size exclusion column and the measured fluorescence intensities were then related to the signal obtained at complete liposome leakage after addition of a solubilizing detergent.

2.5 Immobilization techniques

Lipodisks can be immobilized on solid surfaces by the inclusion of PEG-lipids with functional groups attached to the polymer chain. There are several different functionalized PEG-lipids commercially available and numerous studies report on the diverse usability of these lipids (50, 68-70). By substituting a fraction of the non-functionalized PEG-lipids in the lipodisks with PEG-lipids that have a desired functionality, the disks can be immobilized on various surfaces.

Two coupling methods for lipodisk immobilization that have been used in this thesis are amine and biotin-streptavidin coupling. Bergström et al. (71) previously described a method for protein immobilization through reductive amination on porous silica particles that have been surface-modified into diol silica (71). A similar protocol can likely also be applied for the immobilization of amine-functionalized lipodisks. Lipodisk immobilization through amine coupling can additionally be performed on gold surfaces following a
method described by Hernández et al. (50). In brief, the gold surface is cleaned and surface-modified using 11-mercaptoundecanoic acid. Amine-functionalized lipodisks are then immobilized on the surface following EDC/NHS activation. Amine coupling enables fast and simple immobilization and residual amine binding sites can effectively be inactivated by the addition of a small amine.

There are, however, some cases where amine coupling might be less ideal for immobilization. This can, for instance, be the case when the disks are used to support membrane proteins and especially when the proteins are incorporated in the disks prior to the immobilization step. Proteins are often rich in amine groups and direct protein immobilization on surfaces via amine coupling is a well-known and frequently employed technique. When studying membrane proteins, it is desirable to keep them unperturbed in a near-native environment and the possibility of direct binding to a solid support therefore imposes a problem. An efficient way of ensuring that coupling takes place via the functionalized PEG-lipids in the disks instead of the protein is to use a highly selective coupling mechanism. The biotin-streptavidin bond is one of the strongest non-covalent bonds known in nature and this interaction is frequently used in biotechnological application requiring high selectivity (72-74). Lipodisk immobilization using biotin-streptavidin coupling can be achieved by first immobilizing streptavidin through amine coupling and then adding the biotinylated lipodisks in a second step.

Lipodisks were immobilized on porous silica particles using both reductive amination and biotin-streptavidin coupling in Paper III. Additionally, amine-functionalized lipodisks were immobilized on a gold surface and used for QCM-D analysis.

In Paper IV, lipodisk adsorption onto bare silica was used in order to investigate the effect of polymer chain length on the immobilization properties. Binding was achieved in this study by simply allowing lipodisks to adsorb to the silica surface at 21 °C. Both porous silica particles and smooth QCM and SPR sensor surfaces were used.
2.6 QCM-D

Quartz crystal microbalance with dissipation monitoring (QCM-D) is used to study changes in mass and viscoelasticity of surface layers. In addition to enabling quantification of adsorbed amounts down to a few nanograms, this technique gives information on the mechanical properties of the adsorbed film. QCM-D is receiving growing attention and the technique has been proven useful in studying a number of different systems (75-77). A strong advantage of the QCM-D technique is that not only does it give the adsorbed amount at equilibrium but it also enables monitoring of mass changes in real time, providing important information on adsorption or desorption kinetics.

The QCM technique uses the inverse piezoelectric effect to induce a mechanical stress in a quartz crystal by the application of an electric field. The applied field causes the crystal to oscillate at a certain frequency and a change in the deposited mass causes the frequency of oscillation to shift. The magnitude of this shift can then be used to determine the change in adsorbed mass. For rigid films that are evenly distributed over the surface and have a smaller mass than the quartz crystal, the adsorbed mass \( \Delta m \) can easily be determined from the change in frequency \( \Delta f \) using the Sauerbrey equation (78)

\[
\Delta m = -xA \frac{\Delta f_n}{n}
\]

where \( x \) is the mass sensitivity constant, \( A \) is the active area of the sensor and \( n \) is the overtone number.

With QCM-D both the frequency shift and the change in dissipation factor can be monitored. The latter is a measure of the damping of the oscillation frequency and is related to the rigidity of the adsorbed layer. From the two measured parameters the adsorbed mass and viscoelastic properties of the adsorbed layer can be determined. Since immobilized lipodisks give comparatively high dissipation, the Sauerbrey equation cannot be used to determine the adsorbed mass in this case. In the work presented in this thesis, a model described by Voinova et al. (79) was instead used to fit the experimental data and determine adsorbed mass. QCM-D was used to determine the amount of adsorbed lipodisks in Papers III and IV. In Paper III, the signal was also used to determine the adsorption of the membrane protein cyclooxygenase to the lipodisks.
3. Results and discussion

3.1 Lipodisks as drug carriers

3.1.1 Peptide-lipodisk interaction

The membrane-perturbing effect of, and permeabilization induced by, the two α-helix-forming antimicrobial peptides melittin and alamethicin was investigated in Paper I. These peptides differ in their physico-chemical properties, as well as in their suggested mode of action on phospholipid membranes (80-82). Melittin is a cationic peptide whereas alamethicin is effectively uncharged at physiological pH. However, both these peptides adopt a linear amphiphilic α-helical structure upon association to lipid bilayers. At low concentrations in the membrane, the peptides will adsorb with their helices parallel to the bilayer. This orientation will cause an area expansion of the lipid head-group region, thus inducing a stress in the membrane. In order to reduce the inflicted stress, peptide translocation therefore occurs at high concentrations, causing the peptides to shift from parallel to perpendicular positioning in the membrane. This reorientation causes openings, pores, in the membrane and the difference between the two studied peptides lies in the molecular organization within these pores. The positively charged melittin forms so-called toroidal pores where the pore interior is lined with both peptides and phospholipids. Alamethicin, on the other hand, is suggested to induce formation of pores that are lined only with peptides in a barrel-stave model (83). A schematic illustration of the molecular organization in the different pore models is shown in Figure 12.

The effect of lipid composition on the membrane perturbation of melittin has been thoroughly studied in the past (37, 38). It has been shown, by Lundquist et al., that melittin has a high propensity to induce and associate to highly curved surfaces, such as the rim of the lipodisks (15). Additionally, an effect of membrane curvature on peptide affinity has also been shown for other membrane-active peptides (84).

In Paper I, we could confirm that high affinity for curved surfaces is a property also shared by alamethicin. Interestingly, in spite of their different properties, we found that both melittin and alamethicin induce membrane perturbation and rupture similarly through the formation of open bilayer structures with very high local curvature.
Melittin adsorption to phospholipid membranes can easily be determined by monitoring the shift in intrinsic fluorescence as the peptide redistributes from water into the membranes (85, 86). This blue shift arises from the change in polarity of the environment surrounding the fluorescent tryptophan residue upon membrane insertion. Alamethicin, on the other hand, lacks fluorescent amino acid residues and therefore an alternative method to determine peptide affinity was used. By employing a leakage assay, set up so that higher peptide affinity for lipodisks compared to liposomes results in reduced liposome leakage, preferential binding to lipodisks could be detected. The peptide affinity to lipodisks composed of POPC:cholesterol:ceramide-PEG<sub>5000</sub> (35:40:25) (shown in Figure 14) compared to liposomes was investigated for three different antimicrobial peptides. The reduction in leakage when peptides were added to liposomes in the presence of lipodisks as compared to liposome leakage in the absence of disks is shown in Figure 15. As hypothesized, similar leakage reductions were observed when alamethicin was used, thus confirming preferential binding to the disks also for this peptide. In fact, liposome leakage was dramatically reduced in the presence of lipodisks for all the investigated peptides. The leakage reduction remained pronounced also when the liposome composition was changed by supplementing the POPC bilayer with 40 mol% cholesterol and/or 10 mol% of the negatively charged POPG, indicating preferential binding to the lipodisks compared to all liposome membranes used in the study.

The results shown in Paper I, that high lipodisk affinity is a common feature for all peptides used in this study, consequently strengthens the idea of lipodisks being suitable as carriers for a large number of membrane-active antimicrobial peptides.
Figure 13. Illustration of the competitive binding assay used in Paper I. By comparing the liposome leakage when peptides are added to a mixture of empty and dye-filled liposomes (left) to the leakage obtained when the peptides are added to dye-filled liposomes in the presence of lipodisks (right), preferential binding to the lipodisks can be detected.
Figure 14. Cryo-TEM micrograph of the lipodisks used in Paper I. The arrow indicates a lipodisk observed edge-on and the arrowhead indicates a lipodisk observed face-on. The disks were composed of POPC/cholesterol/ceramide-PEG\textsubscript{5000} (35:40:25). Bar = 100 nm.

Figure 15. Reduction in leakage obtained after addition of peptide to a mixture of dye-filled liposomes and lipodisks as compared to peptide addition to mixtures of dye-filled and empty liposomes. The liposomes used in the experiments were composed of POPC, POPG/POPG (9:1), POPC/cholesterol (6:4) and POPC/cholesterol/POPG (5:4:1). The lipodisks used are shown in Figure 14 and a schematic illustration of the leakage assay is shown in Figure 13.
3.1.2 Peptide delivery using lipodisks

As mentioned previously, lipodisks have potential as carrier particles for the delivery of several different antimicrobial peptides. This possibility was further explored in Paper II using melittin as the model peptide.

When formulating drugs in a carrier system, it is desirable to keep the concentration of free drug as low as possible whilst ensuring a high amount of substance in the carrier system. The melittin-to-lipodisk mixing ratio needed to best meet these requirements was determined using binding isotherms collected by Lundquist et al. (15).

By monitoring the survival of *E. coli* following the addition of free melittin and lipodisk-associated melittin, we could confirm that the peptide maintains its antibacterial effect after formulation in the disks and that it is able to redistribute from the lipodisks into bacteria and induce cell death (Figure 16).

The membrane-associating nature of antimicrobial peptides imposes a considerable risk of uneven drug distributions upon administration of these drugs in free form. It is likely that peptides will avidly distribute into bacterial membranes and all the free peptides might therefore be rapidly consumed upon their initial contact with bacteria. This could mean that the peptides are over-consumed and have thus become tied up in the membranes of dead bacteria, making them unable to exert their full antibacterial effect.

![Figure 16. Time-kill curves after melittin addition to *E. coli* cells. Survival at each time point is given as the geometric mean ± SEM (n ≥ 4).](image-url)
One way of circumventing this problem could be the use of a formulation that slowly releases the drug over time. The possibility of achieving extended melittin release using lipodisks was therefore investigated in Paper II. In order to see if the melittin-lipodisk formulations maintained their antimicrobial effect for longer periods of time compared to free melittin, an experiment was designed where the melittin formulations were repeatedly exposed to fresh bacterial cultures.

Melittin, either free or in lipodisks, was added to *E. coli* suspensions and the cell viability was determined after 15 minutes of incubation. Re-exposure to bacteria was then performed by centrifugation of the samples and subsequent transfer of the supernatant to fresh bacterial suspensions. Cell-killing was then monitored again and the results are shown in Figure 17.

As can be seen in the figure, there is a pronounced difference in the cell-killing efficiency when the melittin formulations are exposed to a fresh bacterial suspension. Whereas all the antimicrobial effect of the free melittin is already gone after a single exposure to *E. coli*, the lipodisk-formulated melittin also shows antibacterial effect in the second exposure, reducing the number of viable cells to about 0.1% in 120 minutes.

*Figure 17. Time-kill curves of *E. coli* cells showing the prolonged bactericidal effect of the melittin-lipodisk formulation. Melittin (either free or in lipodisks) was incubated with bacteria for 15 minutes and then transferred to fresh bacteria where the remaining antibacterial effect could be monitored. The arrow indicates the second exposure step. Survival at each time point is given as the geometric mean ± SEM (n ≥ 4).*
3.1.3 Stability of disk-formulated melittin

One major problem of administering peptides freely in solution is the rapid enzymatic degradation that they undergo in the human body (39, 87, 88). It is possible that formulation of peptides in lipodisks can offer a way of also solving this problem. As has been shown earlier, melittin associated to lipodisks largely co-resides with the PEGylated lipids at the lipodisk rim (15). The polymer layer covering this region of the disks makes it likely that peptides sitting there will be effectively shielded from proteolytic enzymes in the lipodisk surroundings. Lipodisk formulation may therefore be an excellent solution in hindering enzymatic peptide degradation.

In Paper II, melittin susceptibility to the cleaving enzyme trypsin was studied in order to assess the protective effect presented by formulating the peptide in lipodisks. Trypsin cleaves selectively at the peptide bonds of positively charged lysine and arginine residues of its substrates. Tryptic digestion products are therefore easily identified and trypsin is widely used in different biotechnological processes (89). Digestion was studied by incubation of melittin, either free or in lipodisks, with trypsin at 25 °C. In order to follow the degradation process, several experiments were performed with enzyme deactivation/inactivation at different time points followed by mass spectrometry analyses of the obtained digests.

Confirming the hypothesis of peptide protection, the results shown in Figure 18 reveal dramatically improved drug stability as melittin is formulated in lipodisks. When comparing the mass spectra in Figure 18a and b, a pronounced difference in degradation can be observed. Free melittin is rapidly degraded into smaller fragments (Figure 18a) whereas the lipodisks offer excellent peptide protection and disk-formulated melittin remains intact throughout the entire experiment (Figure 18b).

Previous studies of melittin in phospholipid bilayers showed that, although the peptide was associated to the membrane, all theoretical cleavage sites were still accessible for trypsin cleavage (90, 91). It is therefore reasonable to suggest that the protection against peptide degradation offered by the lipodisks is achieved through the steric repulsion from the surrounding PEG chains rather than the membrane insertion of the peptide. It is likely that other degrading enzymes will be hindered from coming into close contact with peptides in the disks in a similar way and the protective effect of lipodisks may therefore be general.
Figure 18. Mass spectra showing melittin degradation after incubation with trypsin for 2.5, 5, 10, 20 and 40 min. (a) free melittin and (b) melittin in lipodisks. The peak labelled a₁ corresponds to intact melittin. All peptides (labelled a₁-g₁) are listed in Table 1 in Paper II.

3.2 Immobilized lipodisks as model membranes
3.2.1 Lipodisk immobilization and drug partitioning

As discussed in section 1.2.2, there is a need for improved membrane-mimicking model systems for the analysis of drug partitioning and membrane protein studies. The possibility of developing a stable chromatographic system with immobilized lipodisks was studied in Paper III.

Lipodisks were immobilized on porous silica particles using two different coupling mechanisms, i.e., amine and biotin-streptavidin coupling. In order to maximize the surface coverage, lipodisks were prepared using detergent depletion. Disks prepared by this technique are typically very small and are thus more likely to penetrate deep into the pores of the silica particles. The lipodisks used in this paper were composed of POPC/Soy PE/cholesterol/ceramide-PEG₂₀₀₀/DSPE-PEG₂₀₀₀amine in molar ratio (30:28:17:21:4) and the disks had hydrodynamic radii below 10 nm according to DLS. The disk size and structure were similar regardless of whether amine or biotin was used as the functional group and a representative cryo-TEM micrograph is shown in Figure 19.

---

1 For lipodisk immobilization using biotin-streptavidin coupling DSPE-PEG₂₀₀₀biotin was used as the functionalized PEG-lipid
Figure 19. Cryo-TEM micrograph of the lipodisks used for immobilization on porous silica in Paper III. The arrow indicates a lipodisk observed edge-on and the arrowhead indicates a lipodisk observed face-on. The disks were composed of POPC/Soy-PE/cholesterol/ceramide-PEG2000/DSPE-PEG2000amine (30:28:17:21:4). Bar = 100 nm.

The disks were coupled to silica either via reductive amination or via streptavidin-biotin binding. Amine coupling yielded slightly higher surface coverage, which may be partially explained by a difference in the surface accessibility using these two methods. Biotin-streptavidin coupling is performed following a two-step protocol where the protein is first amine-coupled to the silica followed by immobilization of the biotin-functionalized lipodisks. The streptavidin covering of the surface may reduce the mean pore diameter and therefore lead to a smaller fraction of the total surface being accessible to the disks. Another possible explanation for the lower binding of biotinylated lipodisks can be that the number of binding sites is lower for these disks as compared to the amine-functionalized ones.

Due to the higher binding, amine coupling was used to immobilize lipodisks on silica in the development of a chromatographic system. The lipodisks were first immobilized on the silica particles and the material was then packed in steel columns. The performance of the produced system was then assessed by HPLC analysis of 15 drug compounds.

The efficiency, reproducibility and stability of the lipodisk columns were found to be excellent. Comparisons of retention times when drugs were analysed as single injections compared to multi-drug mixtures revealed that the drug partitioning was generally unaffected by the presence of other analytes in the injected sample. The effect of storage was assessed by reanalysing a
number of drugs after 82 days. Drug retention was virtually the same before and after storage and it could be concluded that the developed system had a very satisfactory long-term stability. The high stability of the produced columns is consistent with previous reports on the excellent stability of lipodisks (26).

The HPLC experiments run in Paper III were analyzed using both UV and mass spectrometry detection. Because of high salt sensitivity, MS detection was only used in ammonium acetate buffer whereas UV detection was additionally performed using PBS as the mobile phase. A comparison of drug partitioning to the lipodisks in the two different buffers used is shown in Figure 20. The lipodisks used in Paper III had a net negative charge from the included DSPE-PEG lipids. As expected, the electrostatic interactions between charged drugs and the negatively charged disks become more pronounced when the ionic strength of the buffer is lower. This agrees well with what has been reported earlier by Johansson et al. (26).

The robustness of the chromatography media in combination with the possibility of analysing multiple components simultaneously make the HPLC-MS system developed in Paper III far superior to previously presented immobilized lipodisk systems for drug partitioning studies.

![Figure 20. Comparison of Log Kₐ values obtained for drugs of various charges by using covalently immobilized lipodisks.](image-url)
3.2.2 Protein reconstitution

Apart from its usability in drug partitioning studies, the chromatography system developed in Paper III may also present a good model membrane setup for studies of protein-ligand interactions. In Paper III, the possibility of producing columns with immobilized lipodisks containing the peripheral protein cyclooxygenase (COX) was investigated. Lundquist et al. have previously reported on successful incorporation of COX in lipodisks followed by immobilization of these disks on a streptavidin-covered surface (49). A similar approach could also be used to achieve COX immobilization on the silica particles used in Paper III. However, since this protein is peripheral and hence adheres only to the membrane surface, it is possible to produce and immobilize the model membrane first and then add the protein in a second step. Accordingly, immobilized lipodisk columns were prepared followed by the addition of COX. The protein was allowed to associate with the disks and the total protein amount on the column after rinsing was then determined. The results obtained confirm that COX can be immobilized in situ on the developed chromatographic system. Additionally, QCM-D studies of COX association to immobilized lipodisks confirmed that the protein binds selectively to the disks.

3.3 Reducing the PEG chain length

Several lipodisk applications could likely benefit from a reduction in chain-lengths of the PEG-lipids. Stefanick et al. have showed that the targeting efficiency of liposomes can be improved by the use of shorter PEGs (92). It is plausible that the efficacy of lipodisks used for targeted drug delivery would be affected by the polymer chain-length in a similar way. Additionally, through a reduction in polymer chain length it might be possible to reduce the overall lipodisk size without affecting the volume of the phospholipid disk core. Provided that immobilization of lipodisks is limited by the accessible surface area, such a reduction in size is likely to improve the amount of lipodisks that can be bound to a solid support.

In Paper IV we investigated the possibility of producing lipodisks using shorter PEG-lipids than have been used in the past. The studies were done on mixtures of DSPC and DSPE-PEG with polymer molecular weights of 750 and 1000 Da and the structures obtained in these systems were compared to those in DSPC/DSPE-PEG2000 mixtures.

In addition to globular micelles, DSPE-PEG750 has been reported to form structures of lamellar character at temperatures below 70 \(^\circ\)C (17) and it was therefore interesting to study the effect on aggregate structure from the addition of this lipid. Since lipodisks typically form in mixtures of bilayer-forming lipids and lipids that spontaneously form micelles, it was not obvi-
ous that inclusion of DSPE-PEG_{750} in DSPC membranes would induce lipodisk formation. In fact, both cryo-TEM and DLS characterization of DSPC/DSPE-PEG_{750} mixtures indicated that the inclusion of high amounts of this PEG-lipid does not result in pure lipodisk systems. Examples of this are shown in Figure 21.

![Figure 21. Unweighted apparent hydrodynamic size distribution from DLS (a), and a cryo-TEM micrograph (b) showing the presence of aggregates of different sizes in DSPC/DSPE-PEG_{750} mixtures containing 30 mol\% PEG-lipid. The arrows in (b) indicate the large undefined aggregate structures present in these samples. Cryo-TEM scale bar 100 nm.](image)

The structures formed in DSPC/DSPE-PEG_{1000} mixtures were found to be very similar to what has previously been reported in systems containing DSPE-PEG_{2000} and DSPE-PEG_{5000} (14, 16) and inclusion of 20 mol\% of this PEG-lipid resulted in pure lipodisk systems.

The hypothesis that a reduction in polymer chain length increases the amount of lipodisks that can be immobilized on a surface was confirmed in Paper IV. Passive binding to silica surfaces was quantified using lipodisks composed of DSPC with 20 mol\% of DSPE-PEG_{1000}, DSPE-PEG_{2000} and DSPE-PEG_{5000}, respectively. Immobilization on both smooth silica surfaces as well as on porous silica particles revealed a clear trend of increased binding with reduced polymer chain length (Figure 22).
Figure 22. Amount Total phospholipid amount (both DSPC and DSPE-PEGₙ) immobilized on silica surfaces. The graphs show the amount of lipodisks immobilized on porous Kromasil® silica (a) and on smooth silica surfaces using MP-SPR (b) and QCM-D (c). The lipodisks used were composed of DSPC/DSPE-PEGₙ (80:20) and the different PEG-lipids used are listed on the x-axis.

The difference in binding on smooth silica sensors is likely explained by the fact that shorter PEGs will reduce the volume of the polymer corona surrounding the disks. This reduction in volume may facilitate tighter lipodisk packing at the surface and therefore enable more disks to be immobilized. Additionally, reducing the lipodisk size will increase the possibility for disks to penetrate small pores. Both the proposed mechanisms may serve to explain the improved binding of lipodisks on porous silica particles using shorter PEG-lipids. Comparing figures 22 b and c, it appears that the relative difference in immobilization of lipodisks with different PEG-lipids is smaller in QCM-D than in MP-SPR. This effect can be expected, since the QCM-D technique measures the total mass of the adsorbed surface layer, including any incorporated water molecules. Longer PEG-lipids are likely to bind more water and the difference in adsorbed mass with increasing PEG length will therefore be smaller.
3.4 Lipodisks with low PEG-lipid content

An alternative method for lipodisk production is presented in Paper IV. This method was used to produce lipodisks with much lower amounts of PEG-lipids than has been done previously. Up until now lipodisk formation has been observed only at relatively high PEG-lipid concentrations in the membranes. In our work we could show that sonication of DSPC/DSPE-PEG mixtures at a temperature below the main transition temperature of DSPC results in lipodisk formation by inclusion of as little as 2 mol% PEG-lipid. The lipodisks obtained with this technique are relatively large and when the PEG-lipid content was 5 mol% the disk size remained unchanged for at least two weeks.

As we could show in Paper III, lipodisks have great potential as carriers of amphiphilic antimicrobial peptides. The possibility of using lipodisks as drug carriers was also confirmed recently by Zhang et al. (33). Using the anthracycline drug doxorubicin, they showed that formulation of the drug in lipodisks resulted in both extended in vivo circulation and improved tumour distribution.

High cell specificity of a drug carrier can be achieved by conjugating targeting agents to the surface of the carrier particles. Specific targeting of lipodisks may improve the in vitro distribution of drugs and lead to both reduced side effects and increased therapeutic efficacy. The inclusion of a targeting agent can, however, impose limitations on the number of techniques available for lipodisk production. Many targeting agents are sensitive biomolecules that risk losing activity if they are exposed to harsh conditions, such as exposure to liquid nitrogen temperatures and organic solvents, sometimes used for lipodisk production. The problem of adding sensitive targeting agents has been surmounted in liposome preparations by using a post-insertion technique (93) in which the targeting agent is covalently attached to PEG-lipids and thereafter inserted into pre-made liposomes. By excluding the sensitive targeting agent during production, high biological activity in the final preparation can thereby be achieved. The post-insertion strategy has unfortunately not yet been proven successful in lipodisk systems. The limited possibility of adding more PEG-lipids to lipodisks after production is likely explained by the fact that there is already a very high amount of PEG-lipids included in these structures. Due to the steric repulsion of the polymers, it is unlikely that many additional PEG-lipids can insert at the crowded rim of a lipodisk.

It is reasonable to assume that lipodisks with very low amounts of PEG-lipids will improve on the amount of a targeting agent that can be inserted in the disks after production. The low-PEG lipodisks developed in Paper IV were therefore evaluated concerning their ability to adsorb additional PEG-lipids through post-insertion. The amount of post-inserted PEG-lipids was
compared using two different lipodisk preparations. The employed disks were composed of DSPC/DSPE-PEG\textsubscript{2000} with different lipid molar ratios.

The amount of inserted PEG-lipids was quantified using a radiolabelled targeting agent and the elution profiles of this agent after post-insertion is shown in Figure 23. We could show that preparation of lipodisks with low PEG-lipid content clearly increases the amount of PEG-lipids that can be incorporated in the disks through post-insertion. Lipodisks prepared with 5 mol\% PEG-lipid enabled inclusion of more than twice as much of the added PEG-lipids as compared to disks with 20 mol\% PEG. In the disks with high PEG-lipid concentration, 7.8 percent of the added lipids were incorporated compared to 16 percent in the low-PEG lipodisks.

In fact, there are reasons to suggest that the obtained results are even better than might initially be realized. Compared to small disks, the large lipodisks obtained using low amounts of PEG-lipid will have much lower edge-area. The addition of a targeting agent that will reside mainly at the rim of the disks will therefore lead to this agent being much more concentrated at the rim of the large disks than it would be in smaller disks. Because of this, the density of post-inserted PEG-lipids at the rim of the disks can therefore be expected to be even more than twice as high in the large low-PEG lipodisks.

Figure 23. Distribution of targeting lipids after post-insertion in lipodisks composed of DSPC/DSPE-PEG\textsubscript{2000} in molar ratio 95:5 (solid line) and 80:20 (dashed line). Lipodisks elute as a first peak approximately between fraction 20 and 33 (depending on disk size) and micelles of unincorporated PEG-lipids elute as a peak centred around fraction 41.
4. Concluding remarks

The results presented in Papers I and II confirm that lipodisks are promising as drug carriers. The beneficial effects of peptide formulation in lipodisks presented in Paper II, in combination with the fact that all peptides investigated in Paper I displayed high lipodisk affinity, open up for the possibility of using lipodisks as carriers of biologically relevant antimicrobial peptides. The development of new peptide-lipodisk formulations may, however, require some optimization of the lipodisk. Additionally, in order to verify sufficiently high drug-loading capacity, further studies are needed to determine the partitioning of the peptide to the disks. Such partitioning studies can, for example, be conducted using surface immobilized lipodisks in QCM-D as described by Hernández et al. (50).

In Paper III, we successfully developed a stable chromatographic system with immobilized lipodisks. As we show in Paper IV, improved surface immobilization of lipodisks can be achieved by the use of shorter PEG-lipids. Further improvement in the amount of lipodisks that can be incorporated in the chromatographic media can therefore be anticipated by immobilization of disks with shorter PEG-lipids. It should be noted that the positive effect on immobilization observed in Paper IV has not yet been confirmed using functionalized lipodisks. It is likely that shortening the PEG-lipids will have similar benefits on immobilization using functional groups, but additional experiments are needed to confirm this theory. Higher lipodisk immobilization might also benefit the development of chromatographic systems for studying membrane protein-ligand interactions. It is likely that more protein can be immobilized on the chromatography media if the amount of lipodisks is increased.

In Paper IV, we present evidence for the possibility of improving lipodisks in terms of both PEG length and PEG-lipid amount. We show that, apart from improving lipodisk immobilization on porous silica particles, shorter PEG-lipids increases the amount of lipodisks that can be immobilized on smooth surfaces. These findings support the possibility of using shorter PEG-lipids to improve surface immobilization of lipodisks in several different applications.

The new method for lipodisk production presented in Paper IV opens up for the ability to produce lipodisks using very low PEG-lipid amounts. We show that reducing the PEG-lipid amount in the disks increases the amount of PEG-lipids that can be inserted in the disks via post-insertion. This may
prove beneficial in applications where the amount of post-inserted PEG-lipids is crucial, such as cell targeting using drug-carrying lipodisks. Furthermore, the lipodisks prepared using 5 mol% PEG-lipid were comparatively large and thus have low edge-to-bilayer ratios. Large lipodisks with low amounts of edge could provide advantages both in the possibility of achieving higher amounts of surface-immobilized lipids as well as reducing the contribution of the lipodisk rim in drug partitioning studies.

En av huvudkomponenterna i alla biologiska membran är fosfolipider. Fosfolipider tillhör en klass av molekyler som kallas amfifiler. En amfifil molekyl kännetecknas av att den både har en del som är fettlöslig, hydrofob, och en del som är löslig i vatten, hydrophil. Amfifila molekyler kallas även ytaktiva substanser och ritas ofta som molekylen i Figur 1. Andra vanligt förekommande namn på dessa molekyler är surfaktanter, tensider eller detergenter.

![Figur 1. Schematisk bild på en amfifil molekyl.](image)

Om olja och vatten blandas så kommer systemet ganska snart att fasseparera, d.v.s. dela upp sig i två olika faser. På ett liknande sätt kommer den hydrofoba delen av en amfifil molekyl att drivas bort från vatten. Men på grund av amfifilens vattengillande del kommer systemet inte att dela upp sig i två skikt. I vattenlösningar av amfifila molekyler bildas istället aggregat där de hydrofoba delarna vänds bort från vattnet och bildar en fet kärna som omges av molekylernas hydrofila huvudgrupper. Denna process kallas självassociation. Vid vilken koncentration amfifiler börjar självassociera och vilken typ av strukturer som bildas beror av ett flertal olika parametrar. Saltkoncentrationen samt storleken på de hydrofila resp. hydrofoba inslagen påverkar när associationen börjar och strukturen på de bildade aggregaten beror till stor del på de amfifila molekylernas form.
Arbetet i den här avhandlingen har fokuserat på en typ av självassosierte fosfolipidaggregat som kallas lipiddiskar. Diskarna är små (ca 20-200 nm i diameter) stabila aggregat som tål både lång lagring och kraftig utspädning. Lipiddiskar bildas i vattenlösningar där fosfolipider som spontant bildar platta bilagerstrukturer blandas med fosfolipider som har en stor vattenlöslig polymerkedja (PEG) förankrad vid sin hydrofila huvudgrupp, s.k. PEG-lipider. På grund av den stora polymerkedjan bildar PEG-lipider spontant strukturer med hög kurvatur, s.k. miceller. I lipdiskarna kommer de olika komponenterna att segregera så att de bilagerbildande lipiderna sitter på diskens platta del och PEG-lipiderna huvudsakligen befinner sig vid den krökta kanten. En schematisk bild av diskstrukturen visas i Figur 2.

Figur 2. Schematisk illustration av en lipodisk och de ingående lipidtyperna. I det övre vänstra hörnet visas den plana bilagerstrukturen som vanligtvis bildas i rena fosfolipidsystem. I det övre högra hörnet visas strukturen på en globulär micell som bildas i vattenlösningar med bara PEG-lipider. Blandningar av dessa lipider resulterar i lipodiskstrukturen som visas längst ned i figuren.
Lipodiskar har många egenskaper som gör dem till lämpliga kandidater både som bärare av läkemedel och som modellsystem för biologiska membran. I den här avhandlingen har vi utvärderat lipodiskarnas lämplighet för båda dessa applikationer. Vi har dessutom utvecklat en ny metod för att tillverka lipodiskar och studerat möjligheten att ytterligare optimaera diskarna med avseende på både PEG-kedjelängd och mängden PEG-lipid.


I delarbete I och II visar vi att lipodiskar är lämpliga bärare av en typ av bakteriedödande molekyler som kallas antimikrobiella peptider. Antimikrobiella peptider utövar sin effekt genom att förstöra cellmembranet hos de angripande organismerna och de har därför potential att används som alternativ till konventionell antibiotika. Tyvärr finns en del hinder för utvecklingen av dessa peptider som läkemedel. Bland annat är de känsliga för enzymtisk nedbrytning och de skulle därför kunna tjäna på att formuleras i en skyddande partikel, såsom t.ex. lipodisk.

I delarbete I studerade vi tre antimikrobiella peptider och visade att samtliga dessa peptider väldigt gärna binder till den krökta kanten på lipodiskarna. Detta trots att de studerade peptiderna har väldigt olika egenskaper och det är därför sannolikt att en liknande preferens för diskarna kan delas även av andra antimikrobiella peptider. I delarbete II visade vi att en peptid som formulerats i lipodiskarna förtydlande har antimikrobiell effekt samt att peptiden frisätts från disken långsamt och därför kan döda bakterier under längre tid jämfört med peptid som ges i fri form. Slutligen visade vi att formulering i lipodiskar effektivt skyddar peptiden från att förstöras av ett nedbrytande enzym.

En annan mycket viktig anledning till att det behövs bra modeller av biologiska membran är alla de viktiga proteiner som finns i dessa membran. Membranproteinerarna fyller ett stort antal funktioner och många av dem är intressanta mål för läkemedelsbehandling. För att studera proteiner i biologisk membran behövs förenklade modellsystem. Även här har diskarna stor potential och i delarbete III visade vi att det är möjligt att sätta fast ett membranprotein i diskarna när de sitter fast i kolonnen.

I delarbete IV fokuserade vi på att ytterligare optimera lipodiskarna både för applikationer som läkemedelsbärare och modellmembran. Vi visade att det är möjligt att använda kortare polymerkedjor på PEG-lipiderna än vad som används tidigare och att det går att sätta fast fler lipodiskar på en yta om man använder kortare PEG. Att fler diskar fäster på ytan beror troligtvis på att polymerlagret runt diskarna blir mindre med kortare PEG och att diskarna helt enkelt därför kan packas tättare. Vi utvecklade även en ny metod för att tillverka lipodiskar. Den nya metoden gör det möjligt att producera diskar med mycker mindre mängd PEG-lipid än vad som tidigare varit möjligt.

Slutligen visade vi att lipodiskar med liten mängd PEG-lipid är fördelaktiga då man vill sätta till ytterligare PEG-lipider till de färdiga diskarna. Detta kan till exempel vara väldigt viktigt för känsliga målsökarlipider som annars riskerar att bli förstörda under preparationen.
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References


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