Development of lipodisks as carriers for cationic amphiphilic peptides

KARIN REIJMAR BRUNNSTRÖM
Antibiotics have made a tremendous contribution to mankind. They are one of the most successful medicines in human history. However, more and more bacterial strains develop resistance and the risk to public health can hardly be overstated. New types of antibiotics are urgently needed. Antimicrobial peptides (AMPs) have emerged as potential antibiotics because of their broad-spectrum activities and non-conventional mechanism of action. More recently, they have also received attention as promising anticancer agents. The clinical and commercial development of AMPs as a new generation of antibiotics and anticancer drugs is hampered, however, by issues concerning the toxicity, specificity and stability of the peptides.

The aim of this thesis has been to explore if formulation in a novel type of nanocarriers, referred to as lipodisks, can be used to increase the therapeutic potential of AMPs as antimicrobial and anticancer agents. Focus has been on AMPs classified as cationic amphiphilic peptides.

Encouragingly, the data presented suggests that the therapeutic potential of the AMP melittin as an antimicrobial and anticancer agent can be substantially increased by formulation in lipodisks. When formulated in the lipodisk, melittin is protected against enzymatic degradation. The lipodisk also offer a slow-release effect that sustains the bacterial cell-killing effect. We also show that specific delivery of melittin to tumour cells can be obtained by formulating the peptide in small EGF-targeting lipodisks.

Melittin contains a tryptophan residue and its interaction with lipodisks can be characterized by means of fluorimetric binding assays. In order to investigate the binding behavior also for peptides that lack intrinsic fluorescence, we developed a method based on measurements using the QCM-D technique. Studies using this, and other techniques, confirmed that it is a general behavior for cationic amphiphilic peptides to preferentially bind to the highly curved rim of lipodisks. Results of our binding studies show that the peptide to lipid ratio in the lipodisks can be tuned and optimized by varying the size and charge of the disks.

Taken together, the findings in this thesis point towards PEG-stabilized lipodisks as promising nanocarriers for antibacterial and anticancer peptides.
Vad vore jag utan dina andetag...
List of Papers

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


V Ahlgren S.*, Reijmar K. * and Edwards K., (2016) EGF-targeting lipodisks for specific delivery of cationic amphiphilic peptides to tumour cells. *manuscript*

(*) First authorship is shared by these authors

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Paper not included in this thesis:

Comments on my contribution:
Paper I: I was involved in the study design and writing process. Performed the binding assays and participated actively in the evaluation of the data.
Paper II: I was involved in the study design and writing process. Prepared material that was used in the bacterial activity experiment. Performed binding assays and participated actively in the evaluation of the data.
Paper III: I was involved in the study design and writing process. Performed both fluorescence and QCD-D measurements. Participated actively in the evaluation of the data.
Paper IV: I was involved in the study design and wrote the first draft of the manuscript. Performed both fluorescence and QCD-D measurements, participated actively in the evaluation of the data.
Paper V: I was involved in the study design and writing process. Performed binding assays. Helped out with cell experiments. Participated actively in the evaluation of the data.
Contents

1. Introduction ................................................................. 9
   1.1 Antimicrobial peptides ................................................. 9
       1.1.1 Suggested mechanism of action .......................... 12
   1.2 Lipodisks ................................................................. 13
   1.3 Interaction between AMPs and lipodisks ................. 14

2. Experimental techniques ............................................... 15
   2.1 Lipodisk preparation ................................................... 15
   2.2 CryoTEM ................................................................. 16
   2.3 DLS ................................................................. 17
   2.4 QCM-D ................................................................. 18
   2.5 Fluorescence spectroscopy - binding assay ............. 19
   2.6 Association isotherms .............................................. 20
   2.7 Fluorescence spectroscopy - leakage assay ............ 21

3. Results and discussion ................................................ 22
   3.1 Peptide-Lipodisk interaction .................................... 22
   3.2 Peptide delivery using lipodisks ............................... 24
   3.3 Melittin stability in lipodisks .................................... 26
   3.4 QCM-D as a new method to investigate peptide-membrane interaction .............................................. 27
   3.5 Optimization of lipodisks as carriers of peptides .... 30
   3.6 Targeted lipodisks for delivery of ACPs to tumour cells 34

4. Conclusion ................................................................. 38

Svensk sammanfattning .................................................. 39

Acknowledgements ........................................................ 42

References ......................................................................... 44
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACP</td>
<td>Anticancer peptides</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>cer-PEG&lt;sub&gt;n&lt;/sub&gt;</td>
<td>N-palmotoyl-sphingosine-1-(succinyl([methoxy(polyethylene glycol)ₙ])</td>
</tr>
<tr>
<td>CF</td>
<td>5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>cryo-TEM</td>
<td>Cryo-transmission electron microscopy</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DSPC</td>
<td>1,2-distearoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol-3-phosphatidylcholine</td>
</tr>
<tr>
<td>DSPE-PEG&lt;sub&gt;n&lt;/sub&gt;</td>
<td>1,2-distearoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-ₙ]</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol-3-phosphatidylcholine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol-3-phosphatidylglycerol</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz crystal microbalance with dissipation monitoring</td>
</tr>
<tr>
<td>SHM</td>
<td>Shai-Huang-Matsuzaki model</td>
</tr>
</tbody>
</table>
1. Introduction

Antibiotics have made a tremendous contribution to mankind. They are one of the most successful medicines in human history. Not only do they protect us from life-threatening infections, antibiotics also make it possible to perform invasive surgical procedures, deliver pre-term births and treat cancer- and transplantation patients that need to go through immunosuppressive therapies (1).

However more and more bacterial strains develop resistance. The risk to public health posed by increasing antibiotic resistance can hardly be overstated (1). The World Health Organization ranked antibiotic resistance a priority and encourage the development of new types of antibiotics (2). In the early 1980’s, Hans Boman reported that silk moths (Hyalophora cecropia) contained peptides with antimicrobial activity, and that these peptides were potent and had a broad spectrum of activity (3). Michael Zasloff and Robert Lehrer also independently isolated and purified peptides (4, 5). This is the origin of the field of antimicrobial peptide research.

1.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are central components of the innate network of gene-encoded proteins and peptides that protect animals from microbial, viral, or cellular intruders. And they have developed over eons (6). The AMPs found naturally in living organisms are also defined as host defense peptides because of their essential role in constituting the innate immunity system (7). AMPs have been considered potential therapeutic sources of future antibiotics because of their broad-spectrum activities and mechanism of action that differ from conventional antibiotics. More recently, they have also received attention as promising anticancer agents (8). Although they possess considerable benefits as a new generation of antibiotics and anticancer drugs, their clinical and commercial development still has some limitations due to potential toxicity, susceptibility to proteases, and the high cost of peptide production (7).

Antimicrobial peptides are widely distributed in nature, existing in insects and plants, mammals and non-mammalian vertebrates (9). It is presently known that as early as 2.6 million years ago, long before the appearance of adaptive immunity, a simple nonspecific system of innate immunity existed
Research on antimicrobial peptides has strongly expanded during the last decade, as reflected by more than 2700 natural or synthetic peptides with antimicrobial activity listed in the Antimicrobial Peptide Database http://aps.unmc.edu/AP/main.php (11). Most antimicrobial peptides hold a net positive charge of +2 to +7. The cationic nature comes from an excess of basic amino acids (arginine, lysine and histidine) (12). Many of the AMPs are folded in membrane mimetic environments, making one side more hydrophilic and the other side hydrophobic i.e., an amphiphilic molecule (7). The characteristics of the investigated peptides in this thesis are listed in Table 1. And the focus has been on cationic amphiphilic peptides.

The most common explanation for the selectivity of AMPs for microbes over host cells is the difference in membrane interactions due to differences in exposed anionic lipid content (13). The phospholipids that build the membrane of normal mammalian cells are asymmetrically distributed, with an outer leaflet that is composed of predominantly zwitterionic phosphatidylycholine (PC) and sphingomyelin (SPH). On the other hand the predominant anionic lipids in bacterial membranes are phosphatidylglycerol (PG) and cardiolipin (CL) (14). The positively charged antimicrobial peptides allow preferential binding to the negatively charged outer surface of the bacteria (7).

In the early stages of infection, mediators of innate immunity are brought to the front line of defense to combat the invader and protect the host. Antimicrobial peptides are important in this response as they can directly or indirectly exert antibacterial activity (6). Some AMPs are pre-deployed at barrier sites, including the skin or at places that are vulnerable to direct invasion from our environment, like the respiratory and gastrointestinal organs. Other AMPs can be delivered rapidly when needed to provide reinforcements against foreign attack (6).

Recently, it has become evident that AMPs have a diverse range of functions in modulating immunity that have an impact on infections and inflammation. Interestingly, many AMP also bind selectively to tumor cells. Some of the anticancer activities can be explained by the overall negatively charged membrane exterior of the cancer cells, a characteristic shared by the bacterial membranes (15). A substantial number of AMPs can traverse membrane and induce killing by acting on one or more intracellular targets (9).
Table 1. An overview of the antimicrobial peptides used in this thesis. Amino acids with bold letters have a negatively charge and letters with an underscore represents a positively charged amino acid. (References to the peptides in the table (16-22).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin</th>
<th>Amino acid sequence</th>
<th>Charge at pH 7</th>
<th>Hydrophobic residues (hydrophobicity analysis from <a href="http://peptide2.com">http://peptide2.com</a>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>Honeybee venom <em>Apis mellifera</em></td>
<td>GIGAVLKVTTLGLPALISWIKKRQQ</td>
<td>+6</td>
<td>50.0%</td>
</tr>
<tr>
<td>LL37</td>
<td>Human hCAP18</td>
<td>LLGDFFRKSKEKIGKEFRRIVQRKDFLRNLVPRTES</td>
<td>+6</td>
<td>37.8%</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>African clawed frog <em>Xenopus laevis</em></td>
<td>GIHKFLHSAKKFGKAFVGEIMNS</td>
<td>+3</td>
<td>43.5%</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>Soil fungus <em>Trichderma viride</em></td>
<td>PAAIAQAVAGLAPVAEIQ</td>
<td>-1</td>
<td>77.8%</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>Wasp venom <em>Vespa simillima xanthoptera</em></td>
<td>INLKALAALAKKL</td>
<td>+3</td>
<td>71.4%</td>
</tr>
<tr>
<td>Mastoparan X</td>
<td>Wasp venom <em>Vespa simillima xanthoptera</em></td>
<td>INWKGIAAMAKKLL</td>
<td>+4</td>
<td>64.3%</td>
</tr>
</tbody>
</table>
1.1.1 Suggested mechanism of action

There are several proposed mechanisms for how antimicrobial peptides cause damage to the bacterial membrane, but it is most widely accepted that the membrane is permeabilized by pore formation or less well defined disintegration. This leads to a collapse of the transmembrane electrochemical gradient and to a loss of energy, allowing increased water and ion flow across the membrane with cell swelling and osmolysis as a result (23).

Initially, the peptides adsorb parallel to the surface of the bilayer. This will cause an area expansion of the lipid head-group region and induce stress in the membrane. In order to reduce this inflicted stress, translocation occurs at high peptide concentration, causing the peptides to shift from parallel to perpendicular positioning in the membrane. This reorientation causes openings, or pores. The molecular organization of these pores differs between the suggested models and can be different depending on both the peptide and lipid composition of the membrane. A schematic diagram of the different models is presented in Figure 1. According to the barrel-stave model, the peptides form a pore by interacting with the phospholipid acyl chains, forming a transmembrane helical bundle (24). In the toroidal pore model, the peptides are in continuous contact with the phospholipid head groups while forming a pore by increasing membrane curvature. After initial accumulation parallel to the membrane surface, curvature and thinning is induced (25, 26). In the carpet and detergent-like mechanism, peptides accumulate on the surface. When a critical concentration is reached, membrane destabilization occurs (23, 27). The Shai-Huang-Matsuzaki (SHM) unifying model argues that the carpet and toroidal pore models represent the different stages of the same mechanism of action (28).

Figure 1. Schematic of mechanisms of membrane disruption after adsorption onto the membrane. A. Barrel-stave model B. Toroidal pore C. Carpet and detergent-like D. Shai-Huang-Matsuzaki (SHM) model (picture adapted from Gabernet et al. (28)).
1.2 Lipodisks

Lipodisks are flat, circular bilayer structures (Figure 2). They form as a consequence of partial lipid segregation in mixtures of lipids and polyethylene glycol (PEG)-conjugated lipids. In the absence of PEG-lipids, the hydrophobic edges of the disks would be exposed to the aqueous media and they would either close into liposomes or fuse into large lamellar sheets. Some micelle-forming molecules can accumulate and stabilize the edges (29-31) and the PEGylated lipids have been shown to effectively stabilize these small fragments (31, 32). The polyethylene glycol (PEG) moiety is conjugated to the head group of the phospholipid. As a result of this conjugation, the hydrophilic PEG(chain) increases the effective head-group area of the amphiphilic molecule. As a consequence, the PEGylated lipids want to form a structure with a positive curvature (33). Given that the lipids are in the gel-phase state, or the mixture has been supplemented with cholesterol, the result is a flat lipid bilayer surrounded by a highly curved rim. This segregation has been confirmed experimentally (34) and with molecular simulations (35). Around 20-25 mol% PEG-lipid is normally needed to prepare pure lipodisk preparations, even though the formation starts at 5-10%, where coexistence of lipodisks and liposomes can be found (29, 32). Recently, however, Zetterberg et al. developed a new protocol to prepare pure lipodisk preparations with as low as 2 mol% PEG content (36).

Inclusion of PEGylated lipids is a well-known strategy to increase colloidal stability and circulation time for liposomes, and the lipodisks have shown to have a high colloidal stability (37, 38). This is probably a result of the steric repulsion from the many polymer chains at the rim (39-41). This steric repulsion also provides a long blood circulation time (42). Thermal stability has also been shown; once disks have formed in the gel phase, they remain stable even above the lipid transition temperature (31, 32). Due to the low aqueous solubility of the individual components in the disks, they are also stable upon dilution.

The lipodisks also have other advantages like being non-toxic and biocompatible (42).

Figure 2. A schematic illustration of a lipodisk (PEG polymers excluded for clarity).
1.3 Interaction between AMPs and lipodisks

Previous studies have shown that the cationic antimicrobial peptide melittin has a tendency to both induce and stabilize highly curved membrane surfaces, like the rim of the lipodisks (Figure 3) (34). Melittin is a model peptide with a high affinity for most types of phospholipid membranes, and its association with the membrane is mainly driven by hydrophobic interactions and the formation of the \( \alpha \)-helical peptide structure. Free in solution, the peptide is unstructured. In water solutions, the helix-forming tendency of most peptides is relatively low because peptide hydrogen bonds are in competition with hydrogen bonds to the solvent. In contrast, the helix-forming potential is often considerably higher in hydrophobic environments (43-45).

Lundquist et al. (34) investigated and compared the adsorption behavior of melittin in systems containing three different types of lipid aggregates: liposomes, micelles and polymer-stabilized bilayer disks. The findings indicate that the maximum amount of bound melittin is largely governed by the amount of highly curved area available in the sample.

One of the aims of this thesis has been to investigate if it is a general behavior for cationic antimicrobial peptides to preferentially bind to the highly curved rim of lipodisks. A second important purpose was to study the possibility of using the lipodisks as a carrier for AMPs in order to increase their therapeutic potential as antimicrobial and anticancer agents.

![Figure 3. Schematic illustration of a PEG-stabilized lipodisk shown in cross-sectional view. (With permission from Sara Ahlgren)](image-url)
2. Experimental techniques

2.1 Lipodisk preparation

A major advantage with the lipodisks is that they can be custom-made to fit the application, a large variety of lipids have been tested so far (29, 31, 32, 34, 46, 47). The lipids can have different charges, chain lengths, chain saturations and head-groups. The sizes of the lipodisks, ranging from few dozen to several hundred nanometers, can also be varied using different preparation techniques (40, 48).

All lipodisks discussed in this thesis are prepared starting from a lipid film. The desired lipids are mixed in a glass tube, dissolved in chloroform and dried overnight in a vacuum oven. After the prepared lipid film is dried, it can be treated in different ways:

Hydration
The lipid film is hydrated with buffer, freeze-thawed in liquid nitrogen and then extruded through a polycarbonate filter several times. Hydration results in comparatively bigger, less homogenous lipodisks (49).

Sonication
During sonication the lipid sample is exposed to high energy and as a result of this is dispersed into small lipid fragments (50). They are not in the equilibrium state, but relaxation into larger aggregates will be sterically hindered because of the PEGylated lipids. Sonication results in homogenous lipodisk samples (49). Zetterberg et al. have shown that pure lipodisk samples with low PEG content (down to 2 mol%) can be achieved with sonication at low temperatures. These disks tend to be large (36).

Detergent-depletion
Even smaller lipodisks can be prepared with detergent-depletion (49). The lipid film is solubilized with a concentrated surfactant solution. Using a size exclusion column or bio-beads, the detergent is removed gradually and at the same time the aggregates will fuse and grow.
2.2 CryoTEM

Cryo-transmission electron microscopy is a useful technique for studying the size and shape of phospholipid aggregates in aqueous solution. The technique makes it possible to obtain a direct two-dimensional visualization of the sample. No fixation or staining is needed during sample preparation, which lowers the risk of artefacts (51-53).

A very thin film of the sample is cooled in liquid ethane. This super-fast freezing ensures that the lipid structures do not rearrange during sample preparation. The water molecules in the sample are so rapidly cooled that it is impossible for them to organize into ice crystals and you end up with amorphous solid water samples. As a result of this vitreous state of the water, irradiation electrons are able to penetrate the 10-500 nm thin film of the sample, and it is possible to see the aggregates within the film.

In our laboratory we prepare the samples as follows: A small aliquot of the sample is added to a copper grid covered with a polymer film containing small holes. Excess solution is removed by blotting with a filter paper, leaving a thin film in the holes of the polymer. The whole preparation is conducted in a climate chamber with a high relative humidity and a controlled temperature. Once the sample has been blotted, the grid is rapidly plunged into the liquid ethane, which maintains a temperature of about 100 K, and the sample vitrifies. It is important to avoid water condensation that could form ice crystals on the surface, so the sample is held cold in a nitrogen atmosphere during transfer into the microscope and during the analysis.

The method is suitable for particles in the range between ~4 and ~500 nm. The lower limit comes from contrast limitations and the upper limit is a result of the increased scattering from vitrified water in thick sample films. One possible artefact to take into account is aggregate-sorting according to size. This comes from the fact that the thin liquid film covering the holes is thinner towards the middle. This will increase the probability of finding bigger aggregates near the edges and smaller aggregates towards the middle of each hole. It is important to investigate a large number of micrographs of the vitrified sample to get a true representation of the sample. Another possible artefact, especially when looking at liposomes, comes from the evaporation of water during preparation. This can give osmotic pressure difference between the inside and outside of the liposome and a subsequent water transport from the inside. As a result, the structure of the liposome can change. Water evaporation may also cause particle aggregation. The risk of these effects is reduced by ensuring high humidity. Still, to properly characterize the samples it is important to use cryoTEM in combination with a quantitative analysis technique such as dynamic light scattering.

When viewing cryoTEM pictures it is important to realize it is a 2D image of a 3D sample. Thus, both liposomes and lipodisks may appear as
circular objects and care must be taken when interpreting the pictures (Figure 4).

Figure 4. The two pictures at the top show a schematic representation of how a vitrified sample appears in a two-dimensional projection. The micrograph at the bottom show both liposomes and lipodisks of different orientations.

2.3 DLS

Particles in a solution undergo Brownian motion, thus giving the solution local concentration fluctuations over time. Using the Dynamic light scattering (DLS) technique, the size distribution of small particles in a solution can be determined by measuring the temporal intensity fluctuations of the scattered light (54). Looking over a period of time, the scattered intensity, $I_s$, fluctuates around an average value. At small time intervals, the location of the particles is almost the same and the scattered intensities are highly correlated to each other. This correlation will eventually get lost at longer time intervals. The rate at which the correlation is lost is determined
by the translational motion of the particle and can be described by an autocorrelation function, $g(t)$ that can be fitted in different ways to get the translational diffusion coefficient, $D$. From $D$, the hydrodynamic radius $R_h$ can be calculated using the Stokes-Einstein equation

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

where $k_B$ is the Boltzmann constant, $T$ represents absolute temperature and $\eta$ is viscosity of the sample (55). The Stokes-Einstein equation assumes that the scattering objects are spherical and that only Brownian motion occurs. This is to be taken into account when trying to determine the size of the lipodisks, and there are several models that can be used. The model proposed by Mazer et al. relates the radii of disk-shaped objects $R_{\text{disk}}$ to the hydrodynamic radius (56). $L_{\text{disk}}$ is the thickness of the disks.

$$R_h = \frac{3R_{\text{disk}}}{2}\left\{\left[1 + \alpha^2\right]^{1/2} + \frac{1}{\alpha} \ln \left[\alpha + \left[1 + \alpha^2\right]^{1/2}\right] - \alpha\right\}^{-1}$$

$$\alpha = \frac{L_{\text{disk}}}{2R_{\text{disk}}}$$

### 2.4 QCM-D

Quartz Crystal Microbalance with dissipation monitoring (QCM-D) is a technique that can be used to measure differences in the mass deposited on a surface in real time and also make it possible to learn about the viscoelastic properties of the material. The technique is sensitive and adsorption down to a few nanograms can be monitored. This makes it a very suitable technique for studying the interaction between lipid membranes and peptides.

A QCM-D sensor consists of a thin quartz crystal sandwiched between a pair of electrodes. The sensing surface of the quartz piece can be coated with a thin layer of the desired substrate for the analysis (e.g., gold, silica, etc.). Due to the piezoelectric properties of quartz, an alternating current can excite the crystal to oscillate with a certain frequency. As the current is turned off, the damping or dissipation ($D$) of the oscillation can be followed. From the change in frequency, as a result of the added sample to the sensor, the mass of the layer can be calculated. The dissipative properties say something about the rigidity of the adsorbed film. It is the Sauerbrey equation (57) that describes the relationship between adsorbed mass ($\Delta m$) and the change in frequency ($\Delta f$)
\[ \Delta m = -xA \frac{\Delta f_n}{n} \]

where \(x\) is the mass sensitivity constant (17.7 ng cm\(^{-2}\) Hz\(^{-1}\) at \(f_0 = 5\) MHz), \(A\) is the active area of the sensor surface and \(n\) is the number of the specific overtone. But this equation is only valid for rigid films with small changes in dissipation (\(\Delta D\)). In our lipid systems, where the films can be non-rigid and viscoelastic, another relationship must be considered. Determinations of immobilized mass are based on a model developed by Voinova et al. (58). Depending on the viscoelastic properties of the film, the model states that the shifts in frequency (\(\Delta f\)) and dissipation factor (\(\Delta D\)) will be different for different overtones.

For thin adsorbed films in bulk liquid, the changes in frequency and dissipation can be calculated from the two equations

\[
\Delta f = -\frac{m_df_0}{t_q\rho_q} + \frac{4\pi^2 h_1\eta_w\rho_w\eta_1(nf_0)^3}{t_q\rho_q(\mu_1^2 + 4\pi^2\eta_1^2(nf_0)^2)}
\]

\[
\Delta D = \frac{4\pi h_1\eta_w\rho_w\mu_1nf_0}{t_q\rho_q(\mu_1^2 + 4\pi^2\eta_1^2(nf_0)^2)}
\]

In this work we combine the two equations and up with

\[
\frac{\Delta f}{n} = -\frac{m_df_0}{t_q\rho_q} + \frac{\pi\eta_1(f_0)^2}{\mu_1}(n\Delta D)
\]

Where \(n\) is the overtone number, \(m_d\) is the adsorbed mass surface density, \(f_0\) is the fundamental oscillation frequency, \(t_q\) is the thickness of the quartz crystal, \(\rho_q\) is the density of the quartz crystal, \(\eta_1\) is the viscosity and \(\mu_1\) is the elastic modulus of the adsorbed layer.

If our assumptions hold, the combination of the two equations should show that a plot of \(\Delta f/n\) vs \(n\Delta D\) at different values of \(n\) is in line with a y-intercept equal to \(-(m_d,f_0)(t_q\rho_q)^{-1}\), from which the adsorbed mass surface density can be calculated.

2.5 Fluorescence spectroscopy - binding assay

To look at the binding of peptides to lipid-membranes, the tryptophan emission spectrum can be investigated with a SPEX Fluorolog. Peptides that contain a tryptophan in their amino acid sequence shift their emission...
spectrum when the polarity of the environment is changed. When the free peptide in aqueous solution binds to the hydrophobic lipodisk membrane, the $\lambda_{\text{max}}$ of the emission spectra is shifted from 349 nm to 335 nm (59). From the ratio in emission intensity at 325 nm and 355 nm, an association isotherm can be constructed (60, 61). The experiments are performed in a quartz cuvette and at $\lambda_{\text{ex}} = 280$ nm. The initial solution is concentrated peptide and lipodisks are added in small steps.

2.6 Association isotherms

To analyze the data from QCM-D and fluorescence spectroscopy binding assays, an association model developed by Pérez-Paya et al. (61) was used. This model is given by

$$R_{\text{eff}} = \frac{[P]K}{\gamma_p^{\text{lip}}}$$

where $R_{\text{eff}}$ is the membrane-associated peptide to lipid ratio and $K$ is the affinity constant. The activity coefficient $\gamma_p^{\text{lip}}$ describes deviations from the ideal partition behavior (62). This activity coefficient is given by

$$\gamma_p^{\text{lip}} = e^{w \cdot R_{\text{eff}}}$$

In this equation, $w$ describes electrostatic interactions between peptides associated with the membrane and changes in the mechanical properties of the membrane caused by the peptide. When using the Pérez-Paya model, $\ln([P]/R_{\text{eff}})$ can be plotted as a function of $R_{\text{eff}}$, resulting in a line whose slope corresponds to $w$ and the intercept gives $-\ln(K)$.

For comparison, the data were also analyzed assuming ideal Langmuir behavior. The Langmuir association isotherm is given by:

$$R_{\text{eff}} = \frac{R_{\text{eff(max)}} \cdot K \cdot [P]}{1 + K \cdot [P]}$$

where $R_{\text{eff}}$ is the membrane-associated peptide/lipid ratio and $K$ the affinity constant.

In order to obtain the values of $R_{\text{eff(max)}}$ and $K$, the Langmuir equation was linearized using the Scatchard linearization ($y$ axis = $R_{\text{eff}}/[P]$, $x$ axis = $R_{\text{eff}}$, slope = $-K$, and intercept = $K \cdot R_{\text{eff(max)}}$) and the Langmuir linearization ($y$ axis = $[P]/R_{\text{eff}}$, $x$ axis = $[P]$, slope = $1/R_{\text{eff(max)}}$, intercept = $1/(R_{\text{eff(max)}} \cdot K)$).
2.7 Fluorescence spectroscopy - leakage assay

The permeabilization exerted by the peptide on the phospholipid membrane of liposomes can be studied using the leakage of a fluorescent dye. 5(6)-carboxyfluorescein (CF) is encapsulated, in liposomes, in a high enough concentration to make the dye self-quench. At this high concentration the fluorescence intensity is low (50, 63). When peptides penetrate the liposome membrane CF will leak out in the solution where it will be at low concentration and the fluorescence signal will increase (Figure 5).

The liposomes are prepared in a buffer containing a high CF-concentration. The surrounding CF is removed by changing the buffer outside the liposomes, using a size exclusion column. CF-loaded liposomes are exposed to the peptides, the leakage is recorded and the maximum induced leakage obtained for the different peptides is compared to a complete liposome leakage. The degree of leakage can be calculated using

\[
L(t) = 100\left( \frac{I(t) - I_0}{I_{\text{tot}} - I_0} \right)
\]

\(L(t)\) is the percentage of total leakage monitored over time and \(I(t)\) is the time-dependent intensity after the addition of peptide. The \(I_{\text{tot}}\) intensity from totally leaked liposomes is found after adding the detergent Triton-X.

*Figure 5.* Schematic illustration of the leakage assay. On the left, the CF is encapsulated and due to the high concentration, not fluorescent. On the right, the liposome has leaked out some of the CF and it can be detected.
3. Results and discussion

3.1 Peptide-Lipodisk interaction

In paper I, we investigated and compared three α-helical antimicrobial peptides; melittin, alamethicin and magainin 2 in terms of their affinity for lipodisks. The three peptides differ both in their mode of action and in their effective charge at neutral pH; melittin and magainin 2 are cationic and alamethicin effectively uncharged. All of them are α-helical in the presence of a lipid bilayer.

Melittin with its tryptophan residue can easily be investigated. The adsorption behavior can be followed as the intrinsic fluorescence shifts when the peptide goes from the aqueous buffer to the hydrophobic membrane. Finding the membrane affinity for alamethicin, which does not contain any fluorescent amino acid residue, requires another approach. A leakage assay was designed so that a higher peptide-affinity for lipodisks compared to liposomes would give reduced liposome leakage. This could prove the preferential binding to lipodisks.

We looked at four different liposome compositions: (POPC, POPG/cholesterol, POPC/POPG and POPC/cholesterol/POPG). The lipodisks were composed of POPC/cholesterol/cer-PEG5000. The peptides (melittin, alamethicin and magainin 2) were added to a liposome solution in the presence and absence of lipodisks. See Figure 6, which illustrates the setup. After addition the leakage reduction for each case was compared.
Figure 6. Schematic illustration of the competitive binding assay. Release of the fluorescent CF is decreased when the peptide is added to a mixture of dye-filled liposomes and lipodisks (bottom) as compared to the addition of peptide to a mixture of CF-filled and empty liposomes (top).

Liposome leakage reduction could be seen for all investigated peptides and different liposomes in the presence of lipodisks (Figure 7). We compared all liposome compositions and in all cases the results suggest that the uncharged lipodisks bound most of the peptides. The leakage reduction was pronounced even when the POPC bilayer was supplemented with cholesterol and/or negatively charged POPG.

The competitive studies show that melittin, magainin 2 and alamethicin have high affinity for the lipodisks. The result indicates that a large number of different AMPs are likely to show a high propensity for binding the highly curved rim of lipodisk, making them a very versatile drug-carrier.
3.2 Peptide delivery using lipodisks

If we want to use the antimicrobial peptides as an alternative, or complement to conventional antibiotics, the formulation and administration of the peptides need to be safe and efficient. In Paper II, we investigated the possibility of using lipodisks as carriers for the model peptide melittin.

The gram-negative bacteria *Escherichia coli* (*E. coli*) was used to study the effect of melittin loaded onto the lipodisks and the results were compared to the effect of free melittin. One of the challenges is to separate the effect of free peptide inevitably present in the formulation from the effect of peptide released from disks. In pharmaceutical carrier systems, it is important to keep the free concentration as small as possible. It is not feasible to remove all the free peptide, since peptides would be released from the disks to maintain equilibrium conditions. From a binding isotherm, it is possible to find the optimal peptide/lipid ratio to keep the amount of free peptide low. The melittin/lipodisk mixing ratio used was calculated in paper II from an isotherm collected by Lundquist *et al.* (34).

Before looking at the bacterial effect, we wanted to confirm that the peptide loaded onto the disk could be released and exert its effect on lipid membranes. The study was conducted by monitoring the induced leakage from CF-filled liposomes (the method was explained in the technique part of the thesis) and the data showed that the peptide is being released and was able to induce leakage from the liposomes.

*Figure 7.* Leakage reduction obtained 15 min after peptide was added to a mixture of lipodisks and four different liposome compositions: (POPC, POPC/POPG (9:1), POPC/cholesterol (6:4) and POPC/cholesterol/POPG (5:4:1).
The survival of *E. coli* was monitored after the addition of peptide/lipodisk and free peptide. The antibacterial effect was confirmed, induced cell death could be seen for both melittin formulated in the disk and free peptide (Figure 8).

![Graph showing survival of *E. coli*](image)

*Figure 8.* Bactericidal effect after melittin addition to *E. coli*. Survival at each time point relative to that at the start is given as the geometric mean ± SEM (n ≥ 4).

A slow-release effect of therapeutic drugs is often desirable as it can provide a more evenly distributed drug and over a longer time period. The risk of rapidly consumed peptides, and peptides binding to already killed bacteria, is reduced if the peptide is distributed over an extended period of time. A larger portion of bacteria will most certainly also be affected.

In order to investigate if lipodisks could offer a slow-release effect, we used a protocol where the same formulations (free peptide and peptide/lipodisk) were re-used to treat a batch of new bacteria to see if their antibacterial effect remained. The experiment was performed as follows. Free melittin or melittin formulated in lipodisks was added to *E. coli*. After 15 minutes of incubation, the bacteria were removed by centrifugation of the suspension. The supernatant containing remaining peptide, both free and bound, was re-exposed to new bacteria and the effect was evaluated.

In Figure 9, the survival of the *E. coli* is plotted against time. After the first exposure the effect of free melittin is substantial. But it is gone after the second exposure, unlike in the melittin/lipodisk sample, where there is an effect both in the first and second exposures. This can be interpreted as peptide still being bound to the disk and thus continuing to have effect while free peptide has been consumed by the bacteria.
3.3 Melittin stability in lipodisks

One major problem when trying to develop peptide-based drugs is the enzymatic degradation and an often rapid renal filtration \textit{in vivo}. This makes it difficult to maintain a therapeutically relevant concentration in the body for an extended time period (64). If the peptide could be protected from this rapid elimination many promising new peptide-drugs could be developed. As mentioned before, melittin binds preferentially to the rim of the lipodisks (34). At the rim, the PEGylated lipids create a network of polymers. If the peptide is incorporated in this network it is possibly protected from the enzymes in the surroundings. To investigate this, the lipodisks were in Paper II loaded with melittin and then exposed to trypsin. Trypsin is a cleaving enzyme that selectively cleaves the peptide bonds of arginine and lysine residue. Digested products are well known and therefore easily identified in mass spectra (65, 66). The experiment was performed using trypsin on both free melittin and melittin loaded on lipodisks. The effect was examined after incubation with trypsin in 25 °C at different times after exposure. The effect could easily be seen in mass spectra (Figure 10). The free peptide was digested and after 40 minutes all intact peptide was eliminated. After 40 minutes of exposure melittin in the lipodisks was still intact. This confirmed our theory that the lipodisk are able to protect the peptide from proteolytic degradation.

Figure 9. Time-kill curves after first and second exposure to melittin (either free or in lipodisks). The arrow indicates the second exposure step.
3.4 QCM-D as a new method to investigate peptide-membrane interaction

Fluorescence measurements are well-established methods to monitor peptide-membrane interactions, but for this it is essential that the peptide contains a fluorescent amino acid residue. As a consequence, many of the antimicrobial peptides cannot be investigated in this simple manner. In Paper III, we investigate the possibility of using the quartz crystal microbalance with dissipation monitoring – (QCM-D) to characterize the interaction between lipodisks and AMPs. This led to the development of a reproducible and robust protocol to characterize the binding of lipodisks and peptides.

Extruded lipodisks consisting of DPPC/DSPE-PEG$_{2000}$/DSPE-PEG$_{2000}$amine were prepared. The amine is a functional group that was used to covalently bind the disks to the sensor-surface in the QCM-D. We were able to demonstrate that it is possible to stably immobilize our disks this way, see Figure 11 and 12.
Figure 11. A schematic illustration of lipodisks attached to the QCM-D sensor and the binding of α-helical peptides.

Figure 12. Shows the shift in frequency and dissipation obtained during a QCM-D immobilization of lipodisks.

After the confirmation that the lipodisk can be bound to the sensor surface, association isotherms for the different peptides could be recorded. Peptide solutions with increasing concentration were added to the lipodisks. From the data obtained (see example in Figure 13) in the experiments, association isotherms were constructed.
Figure 13. Mastoparan-X binding to the immobilized lipodisks. The label indicate the additions of peptide with increasing concentration. Between the additions, the system is rinsed with buffer.

Parallel to the QCM-D measurements, the binding of the peptide mastoparan-X was determined using a fluorimetric method. The aim was to compare the results from QCM-D and fluorimetric methods. The isotherms from both methods can be seen in Figure 14 and the result has a similar level of confidence. The maximum amount of bound mastoparan-X differs slightly between the methods $R_{\text{eff(max)}} = 0.089 \pm 0.002$ and $0.092 \pm 0.006$ respectively, but the affinity constant is roughly the same $K = 2.3 \pm 0.4$ and $1.8 \pm 0.5 \mu\text{M}^{-1}$.

Figure 14. Association isotherm for mastoparan-X and lipodisks. Filled squares represents the result from the fluorimetric measurements, open circles comes from the QCM-D experiments.
We also used QCM-D to compare the binding of mastoparan-X and mastoparan to lipodisks. A large difference between the association parameters was found. This indicates a potentially different behavior of peptides with similar primary structure after they have been modified with a fluorescent marker, a method that sometimes is used to monitor the binding behavior of peptides without any intrinsic fluorescence. The advantage of the QCM-D method is that no modification is needed.

We also show the possibility of looking at the kinetics of the binding and de-attachment of peptides. Amount bound versus time can be seen in Figure 15.

![Figure 15. Binding curve (the one on top) and the detachment (bottom) of mastoparan-X to lipodisks.](image)

From our studies, we could conclude that QCM-D is a useful technique for studying the interaction between AMPs and lipodisks. The affinity that can be examined between lipodisks and low molecular weight peptides opens the possibility of studying many different interactions with small molecules.

### 3.5 Optimization of lipodisks as carriers of peptides

It is possible to vary several features when designing your lipodisks as a carrier and the optimal properties for the peptide in question can be chosen according to loading capacity, protection against enzymatic degradation and possibility for controlled release. But in order to do this, it is necessary to
fully characterize and understand the interactions between the peptides and the lipodisks. In Paper IV, we focused on the possible effect of lipodisk size, charge and PEG length on association behavior of peptides.

In this study a combination of fluorimetric and nanogravimetric methods were utilized to characterize the parameters describing and controlling the binding of three peptides; melittin, LL37 and magainin 2. These three peptides are relevant by themselves and, at the same time, illustrate the variations that can be expected with AMPs having different features, like effective charge at neutral pH, primary structure and disrupting mechanism.

We examined the binding of melittin to lipodisks of the same size with different charges. The disk compositions in this experiment were DPPC/DSPE-PEG_{2000} (anionic) and DPPC/cer-PEG_{2000} (non-charged). By comparing the binding isotherms for lipodisks stabilized with 25 mol% lipids, we see that the effect of the charge becomes significant (Figure 16). The reason for this is probably that the cationic peptides will start to repel each other upon binding to the uncharged ceramide disks. In the case of the negatively charged PEG-PE disks, the charge will first be screened and the repellent effect will appear later.

![Figure 16. Association isotherms of melittin and lipodisks of different compositions. The effect of the different amount of included PEG-lipids is shown.](image)

Varied lipodisk size could be obtained by using different amounts of PEGylated lipids. The results showed that decreasing the size of the disks results in increased effective peptide/lipid ratios at saturation, for both charged and uncharged lipodisks. This is a result of increasing edge surface area/planar surface area ratio with decreasing size. Given the same amount of bilayer-forming lipids the edge surface area and thus the number of potential binding sites will increase as the size of the lipodisk decreases. A separate experiment with a bilayer of DPPC formed directly on a silica
sensor was performed and the peptide melittin did not bind at all. This further supports our hypothesis that melittin prefers curved edges.

The effect of PEG-chain length was also investigated. A comparison of the binding isotherm obtained for disks stabilized with cer-PEG\textsubscript{2000} and cer-PEG\textsubscript{5000}, respectively show that lipodisks containing longer chains bind more peptide. This comes from the fact that we get smaller disks and more binding sites available because of an increase in edge surface area per total lipid concentration. No effect on affinity from varied chain lengths could be found for the peptide-lipodisk.

We also compared the different peptides and found that magainin 2 has a lower affinity for the lipodisks compared to melittin. This is not a surprise since melittin has a higher hydrophobicity (Table 1). On the other hand, the amount of peptide that can be loaded onto the disks is much higher for magainin 2, this can be seen in Figure 17 and Table 2.

![Figure 17. Adsorption isotherm of the different peptides LL37, melittin and magainin 2 to DPPC/DSPE-PEG\textsubscript{2000} (75/25 mol\%) lipodisks.](image)
Table 2. Parameters describing the peptide association to DPPC/DSPE-PEG<sub>2000</sub> (75/25 mol%) lipodisks.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K$ (μM&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$R_{eff(max)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>15.13±1.36</td>
<td>0.039±0.0004</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>0.161±0.01</td>
<td>0.40±0.009</td>
</tr>
<tr>
<td>LL37</td>
<td>16.63±6.7</td>
<td>0.026±0.002</td>
</tr>
</tbody>
</table>

To further characterize the peptide-lipodisk interaction, we analyzed the binding and release data for magainin 2 recorded with the QCM-D. The binding vs time curves (Figure 18) were analyzed in order to obtain information about the kinetics.

![Figure 18](image.png)

*Figure 18.* Time-resolved traces of magainin 2 binding and release. The different curves represent different peptide concentrations, 15μM on top, 10 μM in the middle and 3 μM bottom. The solid lines represent the non-linear fit, assuming ideal Langmuir behavior.

From these analyses we could conclude that the release rate is fast, the release constant is $7.83 \cdot 10^{-3}$ s<sup>-1</sup> and the half-life is 88.5s. This data indicates that even though a high loading can be achieved, the utility of this formulation in therapeutic applications is likely limited. The data obtained for melittin could not be analyzed because of a fast binding in combination with a rather large bulk effect and a subsequent slow release.

Although LL37 is a peptide used in a lot of studies (67-69), it is difficult to find association data in the literature. This is probably due to the fact that LL37 binds strongly to a wide range of surfaces. To overcome this issue, we used biotinylated sensors and a peptide-saturated environment when collecting binding isotherms. From the results (Figure 17), we see that disks
get saturated at relatively low concentrations and with low amounts. This is
not necessarily a disadvantage, however, and a therapeutic application could
still be possible for a potent peptide.

From these studies, we could conclude that both the lipodisk and the
peptide properties are important for the loading of AMPs onto lipodisks.
Once again, we confirm that the peptides prefer a highly curved edge to bind
and there is no significant binding to the planar part of the disks. A small
disk is also preferred to obtain a good peptide/lipid ratio. The electrostatic
interactions between the lipodisk edge and the peptides do not contribute to
the affinity, but are important for the amount of binding sites on the lipodisk
edge. The interaction determining the affinity is mostly of a hydrophobic
nature and electrostatic effects are negligible in comparison.

3.6 Targeted lipodisks for delivery of ACPs to tumour
cells

As mentioned in the introduction, many of the AMPs also have an anti-
tumor activity and can be referred to as membranolytic anticancer peptides
(ACPs) (70).

As a consequence of the rising interest in these peptides, a database,
corresponding to the one for AMPs mentioned earlier in this thesis, has been
developed. It is called CancerPPD and is a pure ACP database (71). The
development of peptide based therapeutics is complicated, however,
involving the same problems as for AMPs, like rapid degradation and
hemolysis, as well as the addition of low intrinsic selectivity for cancer cells.

In paper V we investigated the possibilities to deliver melittin, a peptide
with a well-known anticancer effect (72-74), selectively to tumour cells by
means of targeting lipodisks. Melittin has low serum stability and is
hemolytic. Recently Gao et al. showed that the hemolytic effect is reduced in
vivo when melittin is loaded onto lipodisks, while the anticancer effect is still
preserved (75). Melittin does not only exert its effect on the cell membrane
integrity, the peptide also permeate the mitochondrial membrane inside the
tumor-cell and thereby induce release of components that will eventually
promote apoptosis (74). Studies in our laboratory have shown that murine
epidermal growth factor (EGF)-targeting lipodisk bind specifically to tumour
cells (A-431) that overexpress the human epidermal growth factor receptor
(EGFR) (76). These studies also showed that there is EGFR-mediated
acellular internalization of the disks. We speculated that by formulating
melittin in EGF-targeting disks it might be possible to obtain selective
intracellular delivery of the peptide to tumour cells.

We know from our earlier studies that melittin binds with high affinity to
the lipodisks (34, 77). Before looking at the anticancer effect, we
investigated if the EGF-targeting agents interfered with the binding of melittin onto the lipodisks. Small EGF-targeting (Figure 19) and non-targeting lipodisks were prepared by BioBead-assisted detergent depletion. The lipodisks were composed of DSPC/DSPE-PEG<sub>2000</sub>/DSPE-PEG<sub>3400</sub> (80/10/10 mol%). In case of targeting disks, a fraction of the DSPE-PEG<sub>3400</sub> molecules were conjugated to EGF via the distal end of the PEG polymer.

Figure 19. Schematic illustration of a melittin-loaded EGF-targeting lipodisk. The targeting agents can be conjugated to the distal end of the PEG-polymers. (With permission from Sara Ahlgren)

The previously described fluorimetric binding assay was used to determine association isotherms for both EGF-targeting and non-targeting lipodisks. From the isotherms it could be concluded that both types of disks can be loaded with substantial amounts of melittin. The isotherms (Figure 20) were used to calculate the most optimal melittin/lipodisk ratio for the cell experiments.

Figure 20. Isotherms describing the binding of melittin to EGF-targeting (○) and non-targeting (■) lipodisks composed of DSPC/ DSPE-PEG<sub>2000</sub> (80/20 mol%).
3H-DPPC supplemented lipodisks were used to investigate the in vitro cell-binding characteristics of the melittin-loaded disks. The results show that incubation of the cells with EGF-targeting lipodisks results in a significantly higher cell-associated 3H-radioactivity, as compared to incubation with non-targeting disks. As a control, a blocking experiment was conducted in which the cells were pre-incubated with a high amount of free EGF. The blocking lead to a significant reduction of the cellular binding of the targeting lipodisks (Figure 21).

The cytotoxicity of targeting and non-targeting lipodisks loaded with melittin was evaluated by use of the XTT colorimetric cell viability assay. The disk/melittin samples were incubated with the A-431 cells for 3h before substituting the incubation media to fresh cell media. After another 21h the medium was substituted for XTT solution. XTT, a tetrazolium dye, is cleaved by dehydrogenase enzymes of metabolically active cells which yields a highly colored formazan product. This colored derivative can easily be detected in absorbance measurements (78).

The results, shown in Figure 22, indicate that the presence of the targeting agent (EGF) leads to a significantly improved cell-killing effect. The detailed mechanism(s) behind the increased potency of the targeting lipodisks remains to be clarified in future studies. It is possible, however, that EGF receptor mediated internalization of the melittin-loaded lipodisks increases the probability for permeabilization of the mitochondrial membranes. Another explanation could be that binding of the disks to the EGF receptor leads to a more effective permeabilization of the cell membrane.
Figure 22. Cytotoxicity of melittin-loaded targeting and non-targeting lipodisk to A-431 cells evaluated with XTT assay (n=8).
4. Conclusion

One important aim of the studies in this thesis has been to explore the possibilities to use PEG-stabilized lipodisks as carriers for cationic amphiphilic peptides. Encouragingly, the data presented in paper II and V suggest that the therapeutic potential of melittin as an antimicrobial and anticancer agent can be substantially increased by formulation in lipodisks. Thus data in paper II confirmed that the PEG-ylated lipids form a polymer network that protects the melittin from enzymatic degradation. Also, it could be concluded that formulation in the lipodisks allowed for a slow peptide release that sustained the bacterial cell-killing effect. Moreover, the results presented in paper V showed that specific delivery of melittin to tumour cells can be obtained by formulating the peptide in small EGF-targeting lipodisks.

When developing lipodisk-based formulations it is important to optimize the peptide to lipid ratio in the disks. In order to do this the peptide binding behavior needs to be characterized. Many AMPs and ACPs lack intrinsic fluorescence and in paper III and IV we show that QCM-D is an excellent technique that can be used to investigate the binding of such peptides to lipodisks. The real-time measurements make it possible to investigate both thermodynamic and kinetic parameters, thus providing with important information for the optimization of the nanocarriers. Importantly, the investigations performed with QCM-D and other techniques in papers I, III and IV suggest that it is a general behavior for cationic amphiphilic peptides to preferentially bind to the highly curved rim of lipodisks.

Taken together, the findings in this thesis point towards PEG-stabilized lipodisks as promising nanocarriers for antimicrobial and anticancer peptides. The knowledge and insights gained from the studies is of important value for the continued development of lipodisk-based formulations of antimicrobial peptides into safe and effective drugs.
Svensk sammanfattning

Det senaste århundradet har mänskligheten haft stor nytta av antibiotika för att behandla livshotande infektioner. Antibiotika har även haft avgörande betydelse vid t.ex. operationer, för transplantationspatienter och cancerpatienter där behandlingen kan innebära en ökad infektionsrisk.

Dessvärre utvecklar fler och fler bakteriestammar resistens (motståndskraft) mot konventionell antibiotika. Konsekvensen av denna utveckling är att våra nuvarande skydd mot infektioner riskerar att bli verkningslösa i framtiden och att mänskligheten snart kan befinner sig i samma position som för hundra år sedan i kampen mot bakterierna. Världshälsoorganisationen (WHO) ser denna snabba resistensutveckling som ett av de största hoten mot vår hälsa och uppmanar till forskning som kan leda till upptäckter av nya typer av antibiotika. En möjlig lösning skulle kunna vara de antimikrobiella peptider (AMPs) som är en del av vårt medfödda immunförsvar och som har utvecklats genom evolutionen under tusentals år. De antimikrobiella peptiderna förekommer i många varierande former i både växter och djur. Och fungerar som ett skydd mot hot utifrån i form av bl.a. bakterier och virus. På senare tid har man insett att antimikrobiella peptider skulle kunna användas som en ny typ av antibiotika. Peptiderna har effekt mot många typer av infektioner och verkar genom en mekanism som skiljer sig från de antibiotika som används idag. Förutom deras lovande antibakteriella effekt har det nyligen också visat sig att flera antimikrobiella peptiderna kan användas för att bekämpa cancerceller. Intresset för detta forskningsområde är mycket stort och idag finns fler än 2700 peptider i databasen The Antimicrobial Peptide Database.

En av fördelarna med de antimikrobiella peptiderna är att de inte skadar friska celler utan främst utövar sin effekt på bakterier eller cancerceller. Detta beror på att höljet (membranet) som omger bakterier och cancerceller har en annan uppbyggnad och andra egenskaper än höljet runt våra vanliga celler. Effekten av peptiderna anses vara att de gör så att det bildas porer (hål) i dessa membran. När det bildas en por kan bakterien/cancercellen inte längre upprätthålla sin livsviktiga inre miljö vilket slutligen leder till att den dör. Jämfört med verkningsmekanismen hos de antibiotika som används idag är denna verkningsmekanism mycket svårare för cellerna att utveckla resistens mot.

Det är emellertid inte helt okomplicerat att använda antimikrobiella peptider som läkemedel. En utmaning med dessa peptider är den snabba
nedbrytning de ofta genomgår i kroppen. En möjlig väg runt detta problem kan vara att man binder peptiden till en större struktur där den skyddas från nedbrytning undertiden den transporteras in i kroppen och ut i blodet. I denna avhandling undersöker vi om lipodiskar skulle kunna användas just som en sådan bärare.

Lipodiskarna är uppbyggda av lipider, en typ av fettmolekyler som bl.a. bygger upp membranhöljet runt våra celler. Till dessa lipider kan man koppla en stor vattenlöslig polymer som kallas polyetylenlykols (PEG). Om man blandar lipider med och utan PEG-kedja kan resultatet bli att molekylerna associerar till små diskformade aggregat (Figur 1) med en storlek på 20–200 nm i diameter. Denna diskform uppkommer eftersom lipiderna som inte har en PEG-kedja föredrar att sitta i en plan struktur medan de lipider som har en PEG-kedja istället föredrar att befina sig där strukturen är böjd. Lipodiskarna har många egenskaper som gör dem till lämpliga bärare av läkemedelsmolekyler. Diskarna är mycket stabila och beredningar kan både spädas och förvaras länge utan att diskstrukturen förändras.


De resultat som presenteras i denna avhandling visar att de PEG-stabiliserade lipodiskarna har egenskaper som gör dem mycket intressanta som bärare av antimikrobiella peptider och att det finns stort värde i fortsatta studier av lipodiskar för denna applikation.
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References


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)

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