Interaction Between Antimicrobial Peptides and Phospholipid Membranes

Effects of Peptide Length and Composition

LOVISAN RINGSTAD
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Abstract

Due to increasing problems with bacterial resistance development, there is a growing need for identifying new types of antibiotics. Antimicrobial peptides constitute an interesting group of substances for this purpose, since they are believed to act mainly by disrupting the bacterial membrane, which is a fast and non-specific mechanism. In order to understand the details on this action simplified phospholipid model membranes based on liposomes, monolayers and bilayers, were employed in this thesis.

By in situ ellipsometry studies on supported lipid bilayers in combination with leakage from liposomes it was found that peptide-induced membrane rupture to a great extent is related to peptide adsorption. The peptide activity and mechanism of action is highly dependent on peptide properties such as length, topology, charge, and hydrophobicity. Electrostatic interactions are crucial for peptide adsorption, whereas α-helix formation is of less importance, demonstrated by the dominating peptide conformation being random coil both in absence and presence of membranes, as investigated by circular dichroism. Comparable effects were observed in both mono- and bilayer systems, showing that formation of transmembrane structures is no prerequisite for membrane rupture by complement-derived peptides. Electrochemical studies on these peptides further demonstrated that hydrophobic interactions facilitate peptide penetration into the membrane, causing defects in close proximity to the peptides, while strong electrostatic interactions arrest the peptide in the headgroup region. Increasing the peptide hydrophobicity, by e.g., tryptophan end-tagging, also increases salt resistance.

Good correlations were found between model membrane investigations and antibacterial activity towards both Gram-negative and Gram-positive bacteria, showing that membrane rupture is a key mechanism of action for the peptides investigated. In addition, for all peptides investigated cell toxicity is low.

Keywords: Adsorption, antibacterial, antimicrobial peptide, bilayer, ellipsometry, electrochemistry, electrostatic interactions, hydrophobicity, liposome, membrane, monolayer, phospholipid, secondary structure, supported bilayer.

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List of Papers

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


V Ringstad, L., Pasupuleti, M., Schmidtchen, A., Malmsten, M. Interaction between W-tagged kininogen-derived peptides and model lipid membranes. *Manuscript*

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My contribution:
Paper I, III and V: I was involved in all parts of the work, i.e., in the study design, all experimental work except for the antibacterial and cytotoxic studies, analyzing the data, and the writing process.
Paper II: I was partly involved in the study design, analyzing the data, and the writing process, and performed the experimental work on adsorption, size, and z-potential.
Paper IV: I was involved in all parts.
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Abbreviations

ac Alternating current
AMP Antimicrobial peptide
C3a Complement component 3a
CF 5(6)-carboxyfluorescein
CD Circular dichroism
CL Cardiolipin
CV Cyclic voltammetry
d Bilayer thickness
DDM $n$-dodecyl-β-D-maltoside
DLS Dynamic light scattering
DOPA 1,2-dioleoyl-sn-glycero-3-phosphate
DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPG 1,2-dioleoyl-sn-glycero-3-(phospho-rac-(1-glycerol))
DPG Diphosphatidylglycerol
ε Dielectric constant
E. coli Escherichia coli
Γ Adsorbed amount
HMDE Hanging mercury drop electrode
HMWK High molecular weight kininogen
κ−1 Debye screening length
LPS Lipopolysaccharide
n Refractive index
P. aeruginosa Pseudomonas aeruginosa
PC Phosphatidylcholine
PCS Photon correlation spectroscopy
PE Phosphatidylethanolamine
PG Phosphatidylglycerol
PS Phosphatidylserine
$R_b$ Hydrodynamic radius
RDA Radial diffusion assay
S. aureus Staphylococcus aureus
SM Sphingomyelin
ζ Zeta potential
ZFC Zero frequency capacitance
One-letter abbreviations used for amino acids

A  Alanine
C  Cysteine
D  Aspartic acid
E  Glutamic acid
F  Phenylalanine
G  Glycine
H  Histidine
I  Isoleucine
K  Lysine
L  Leucine
M  Methionine
N  Asparagine
P  Proline
Q  Glutamine
R  Arginine
S  Serine
T  Threonine
V  Valine
W  Tryptophan
Y  Tyrosine
Introduction

When Alexander Fleming, by mistake, discovered penicillin in his lab in London in 1928 antibiotics was born, and in the 1940s it was used to treat bacterial infections for the first time. The fact that life threatening infections could be overcome was a major breakthrough in medicine, and during the following decades many different types of anti-infective therapeutics were developed (1,2). However, as the use of antibiotics increased so did development of resistant bacteria, bringing back the spreading of harmful bacterial infections, despite increasingly advanced and potent drugs being developed. Because of this, there is a growing need for new types of antibiotics. Interesting in this context is the innate immune system, that protects the body from invading pathogens and most of the time prevents infections. In particular, antimicrobial peptides (AMPs), or host defense peptides, serve as a first line of defense against invading pathogens. These peptides constitute a group of substances of interest for designing future antibiotics since they have several properties which are advantageous for this purpose. AMPs have been well preserved during evolution and act mainly by breaking down the bacterial membrane, a rapid and non-specific mechanism that may complicate resistance development (3-5). Being non-metabolic, AMP action is typically fast, of interest for rapidly progressing infections. In addition, many AMPs are low in toxicity towards eukaryotic cells. Although affecting many different parts and functions of bacteria, the main target of these peptides is the bacterial membrane. In order to investigate how AMPs act on bacterial membranes, studies on simplified model membranes can be employed, which will be the main focus of this thesis.

Antimicrobial peptides

Origin and general characteristics

Antimicrobial peptides are a part of the innate immune system and are rapidly upregulated when encountered by pathogens. AMPs were first discovered in the 1970s by Boman et al in Drosophila flies (6). After that, a large number of new substances have been discovered, originating from plants, animals, and human (4,7-9), and to date over 1400 AMPs (10) have been identified, including synthetic AMP mimics (11-13). In humans, defensins
and cathelicidins (LL-37) are the classical AMPs, present in high concentration in tissues frequently exposed to pathogens, such as skin, lungs, and the gastrointestinal tract. The activity of AMPs towards bacteria has been subject of considerable research, but AMPs are also active against fungi, protozoa, and even cancer cells and some enveloped viruses (14-16). For simplicity, the peptides will be discussed in terms of antibacterial properties in this thesis, although the reasoning may be at least partly valid also for other types of pathogens.

In general AMPs are cationic and amphiphilic, containing both hydrophilic and hydrophobic parts that are arranged in different segments of the molecules. AMPs are rather small, normally composed of 10-40 residues, the amino acid arrangement varying greatly between different AMPs. Some AMPs are rich in one type of amino acid, examples being cysteine-rich defensins and histidine-rich histatines, but most contain a variety of amino acids. There is also considerable structural diversity among AMPs, where the most widely discussed ones are those adopting a linear α-helical structure, especially upon membrane interaction. Others contain disulfide bridges (e.g., defensins) resulting in β-sheet formation. There are also linear, more randomly structured AMPs, as is the case for most of the peptides investigated in this thesis.

The majority of the AMPs studied in this thesis (Table 1) originate from human and have been identified during infection-related proteolytic degradation of larger endogenous proteins from the complement (C3a) (17) or contact system (kininogen) (18). In addition to complement and contact peptides, also consensus peptides (Cardin motif), i.e., specific sequences that have been shown to be important for certain recognition, e.g., heparin binding (19), were investigated.
Table 1. Properties of the peptides investigated. Explanations for the one-letter abbreviations used for the amino acids can be found in the abbreviations section.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Net charge$^1$</th>
<th>Origin</th>
<th>Paper</th>
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<td>AKKARA</td>
<td>+3</td>
<td>Cardin motif</td>
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<td>AKK12</td>
<td>AKKARAACKKARA</td>
<td>+6</td>
<td>I</td>
<td></td>
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<tr>
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<td>AKKARAACKKARAACKKARA</td>
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<td></td>
</tr>
<tr>
<td>AKK24</td>
<td>AKKARAACKKARAACKKARA</td>
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<td>I</td>
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<tr>
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<td>ARKKAAKA</td>
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<td>HKH20</td>
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<td>Contact</td>
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<td>HKH10</td>
<td>HKHGHGHGKH</td>
<td>+2</td>
<td>II</td>
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<td>KNK10</td>
<td>KNGGKKKNGKH</td>
<td>+5</td>
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<td>CHK22-l</td>
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<td>CHK22-c</td>
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<td>III, IV</td>
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<tr>
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<td>CNYITELRQQARASKLGLAR</td>
<td>+5</td>
<td>III, IV</td>
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<tr>
<td>CNY21R-S</td>
<td>CNYITELESSQHASASHLGLAS</td>
<td>-1</td>
<td>III, IV</td>
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$^1$ At pH 7.4

$^2$ Cyclic peptide

Plausible mechanisms of action

AMPs have been shown to exert their primary bactericidal action by rupturing the membrane of the target cell, thus causing a fast and non-specific killing. These membrane interacting properties of AMPs are the main focus in this thesis. However, it is important to bear in mind that some AMPs have multiple targets and affect intracellular functions in addition to/instead of the bacterial membrane (20,21), or act through membrane receptors. In addition to directly killing bacteria, AMPs may also act as signaling molecules in the immune system, and by that link innate and adaptive immune responses (9,16,20).

Several models have been proposed for the mechanism of action for membrane disruption by AMPs (4,22,23). The classical ones are based on formation of stable or short-lived membrane pores of the barrel-stave (24) or toroidal (25) type, or bacterial breakdown in a detergent like action, as summarized in Figure 1. According to the barrel-stave model, the peptides form transmembrane pores composed of peptide oligomers where peptides have adopted an amphiphilic $\alpha$-helical structure with the hydrophobic part facing
the hydrophobic bilayer interior. In order to produce these types of pores specific peptide properties are required in terms of size, helicity, and amphiphilicity, which imply that this mechanism is not likely to be very common. Although frequently discussed as a mechanism action, barrel-stave pores have only been experimentally proven to occur for a few peptides where alamethicin (24,26) is the most well-known example. Toroidal pores, on the other hand, can be formed by a greater variety of peptides. Prior to formation of both barrel-stave and toroidal pores, the peptide presumably adsorbs parallel to the membrane surface (27-29). When a certain (local) concentration is reached, the peptide either inserts into the membrane as discussed above, or induces a positive curvature strain in the membrane, which results in an opening, the so-called toroidal pore. Upon further increasing the peptide concentration, or in parallel to toroidal pore formation, two additional scenarios can take place. In one of these, higher peptide amounts on the membrane surface may eventually cause micellization in a detergent-like manner (23), although initial pore formation is not a prerequisite for this action. In a second one, the mass imbalance across the bilayer due to increasing peptide adsorption results in peptide translocation across the membrane to the inner membrane leaflet, which can take place through transient toroidal pores or even without pore formation (30,31). In addition, peptide adsorption in the polar headgroup region causes lateral expansion of the lipid membrane, which allows relaxation of the acyl chains, and results in membrane thinning, further facilitating membrane rupture (32-34).

Depending on the composition of the membrane, also peptide-induced phase transitions (22) or lipid segregation (13,22) may cause membrane rupture. Membrane lipids such as phosphatidylethanolamine (PE), an abundant component of bacterial membranes (as will be discussed in the next section) are sensitive to phase transitions. For example, experimental data have shown that certain peptides induce transitions from lamellar to cubic (35) or inverted hexagonal phases (36) in PE-containing membranes. Segregation of membrane lipids due to for example favorable interactions between cationic peptides and anionic membrane lipids may also be a reason for membrane rupture (37). The domains formed have characteristics that differ from the rest of the bilayer and destabilization occur either due to local defects in the phase boundary between the different domains, or due to lipid packing disorder or membrane thinning around the adsorbed peptide molecules. These events may eventually lead to formation of pores as discussed above and showed in Figure 1.
The mechanism of action is highly dependent on peptide structure and properties, as well as on membrane composition. The mechanisms proposed here present a somewhat generalized picture and it is likely that combinations of different mechanisms take place, and that transitions between the different states occur. As will be discussed in detail in the Results and discussion section, electrostatic and hydrophobic interactions are crucial for membrane rupture.

Membrane organization

Eucaryotic cell membrane vs bacterial membrane

The biological membrane is crucial for bacteria/cell survival and serves as a permeability barrier for transport of molecules in and out of the cell. Mem-
brane rupture therefore result in bacteria/cell death. The central structural elements of cell membranes are phospholipids (38). In addition, membrane proteins are abundant components, constituting up to half of the total membrane weight, and are crucial for many specific membrane functions (38,39). Some membrane proteins as well as membrane lipids have carbohydrates attached, located at the membrane surface where they have a protective function in addition to being important for interactions between cells (38,40).

Eukaryotic cell membranes and bacterial membranes have similar functions but differ in the overall composition, both in terms of phospholipids and in additional components, schematically described in Figure 2. For example, cholesterol is a fundamental part of eukaryotic cell membranes constituting up to 45% of the total lipids (41), while it is absent in bacterial membranes. In addition, there are also considerable differences in phospholipid composition. For example, in the membrane of red blood cells, there are differences in lipid composition between the inner and outer bilayer leaflet, where choline lipids (phosphatidylcholine, PC, and sphingomyelin, SM) are most abundant in the outer leaflet, while the cytoplasmic leaflet mainly contains amino lipids (phosphatidylethanolamine, PE, and phosphatidylserine, PS) (42-44). The lipids are zwitterionic, except for PS being anionic, rendering the outer part of the membrane uncharged (38).

When considering bacteria there are two classes, Gram-negative and Gram-positive, with highly different membrane structures. Gram-negative bacteria have two membranes separated by a layer of peptidoglycan, where the outer membrane is covered by lipopolysaccharides (LPS) anchored to Lipid A in the membrane. Gram-positive bacteria, on the other hand, contain only one membrane covered by a thick polymer network composed of peptidoglycan and teicholic acid. The cytoplasmic membrane is negatively charged due to presence of phosphatidylglycerol (PG) as well as di-phosphatidylglycerol (DPG) (also referred to as cardiolipin, CL). Regarding the type and amount of different membrane phospholipids there are great differences between bacterial strains (45). For example, the E. coli outer membrane is composed of about 91% PE, 3% PG, and 6% DPG, while the cytoplasmic membrane contains 82% PE, 6% PG, and 12% DPG (46). In total, approximately 40% of the fatty acids are saturated, the dominating chain lengths being 16 and 18 carbons (46). S. aureus membranes, on the other hand, contain PG-lipids only, i.e. 36% PG, 7% DPG, and 57% lysylPG (i.e., PG esterified with L-lysine) (47,48). In addition, all fatty acids are saturated and the chain length varies from 14 to 20 carbons (49).
Figure 2. Schematic illustration of the membranes of eukaryotic cells and Gram-negative and Gram-positive bacteria.

Membrane models

As discussed above and shown in Figure 2, bacterial membranes are complex structures. In studies of the interaction with peptides it is therefore difficult to discriminate what parts/functions of the membrane that are involved. In addition, when using bacterial systems also bacterial functions apart from the membrane may influence the peptides. Thus, in order to investigate the details on peptide-membrane interactions simplified model membranes composed of phospholipids alone are advantageous. Although being far from authentic, such membranes allow studies of specific parameters of interest, both in terms of peptide and membrane composition.

There are many different types of membrane models, the main ones being based on liposomes and supported membranes (13,22,50-54). To briefly summarize, peptide-membrane interactions can be studied from a global membrane perspective in terms of, for example, lipid chain ordering and packing, membrane thickness and curvature, and thermodynamic alterations by techniques such as differential scanning calorimetry (DSC), X-ray diffraction, neutron scattering, spectroscopic and nuclear magnetic resonance
(NMR) techniques, microscopy techniques, and many more (33,55-60). Also local membrane features, such as molecular diffusion, flip-flop, peptide localization, and lipid organization around defects can be investigated using e.g., molecular dynamics simulations, spectroscopic methods, and NMR (58,61-64). However, local and global properties cannot be strictly discriminated since these are related and affect each other. Combinations of different methods are thus required to obtain a complete overview. In addition to direct studies of membrane characteristics, examining the peptide properties in the absence and presence of model membranes generates information on peptide-lipid interactions, e.g., from structural alteration of the peptide upon membrane interactions (65). Lipid composition in model membranes reported in literature ranges from one-component to more complex bacterial mimic systems, where the most commonly used lipids are PC, PE, and PG with different chain length and saturation.

In this thesis both liposomes and supported mono- and bilayers have been employed, together with peptide characterization methods as those described in the Experimental section. Some discrepancies in terms of membrane properties between the different models should be noted. For example, the liposomes used are rather large, around 100-150 nm, thus the intrinsic curvature strain is low and they can be regarded as a planar bilayer that, due to the closed structure, mimic the overall structure of cells. In liposomal membranes the lipids are free to move laterally, which is a fast process, and to some extent also flip-flop between the two leaflets (41,66). In addition, exchange of lipid molecules between liposomes occur (66). In the monolayers employed the lateral mobility is likely to be fast, but due to lack of an inner leaflet, no lipid flip-flop takes place. Regarding supported bilayers the lipid lateral mobility is dependent on the deposition technique used for bilayer formation. In bilayers formed by the Langmuir-Blodgett technique, the lateral diffusion is similar as in liposomal membranes (67). The supported bilayers employed here, on the other hand, deposited from mixed micelles and liposomes as described in the Experimental section, are rather tightly attached to a substrate mainly by van der Waals forces (68) with only a small hydration volume (~less than 5Å) separating the bilayer from the substrate (69,70). This is expected to reduce the lateral mobility in the inner leaflet to some extent and also the flip-flop rate in comparison to liposomes. In addition, the membrane properties are of course also highly influenced by the membrane composition, as well as presence of additional components, such as peptides.

The phospholipids used in this thesis (Figure 3) are of the dioleoyl type, which means that their hydrophobic part is composed of long, symmetrical, and unsaturated acyl chains that allow formation of stable and well defined liposomes and mono- and bilayers that are in fluid state in a wide temperature range (71). By mixing phospholipids with different head group charges
while keeping the acyl chains unchanged, the effect of membrane electrostatics alone can be studied.

Figure 3. Molecular structures for the phospholipids and cholesterol used in the model membranes.

In paper I-III zwitterionic and anionic bilayers were employed composed of DOPC/cholesterol (60/40) or DOPC/DOPA/cholesterol (30/30/40), respectively. These compositions were not chosen to mimic general functions of the bacterial membranes, but merely to generate stable and well-defined bilayers with different charge densities in order to study electrostatic interactions. In paper IV DOPC membranes without cholesterol were employed to obtain monolayers suitable for electrochemical studies. Cholesterol-free membranes were used also in Paper V, where pure DOPC membranes were employed together with membranes composed of lipids that further mimic those included in bacterial membranes (DOPE/DOPG and \textit{E. coli} lipids).

In order to gain a more biologically relevant picture of the interactions and whether the bacterial membrane is involved in the mechanism of action for the peptides investigated, studies on model membranes were compared to antibacterial and cell toxicity investigations throughout.
Aims of the thesis

The main goals of this work were:

• To further clarify the mechanism of action of antimicrobial peptides of different origin by use of model phospholipid membranes with focus on peptide adsorption density.

• To elucidate how peptide properties such as length, composition, secondary structure, and topology affect the peptide interaction with lipid membranes.

• To generate new knowledge on the importance of electrostatic and hydrophobic interactions for peptide adsorption and membrane rupture.

• To correlate membrane disruptive properties to antibacterial and cytotoxic activity.
Experimental techniques

Ellipsometry

The technique used for studies on peptide adsorption to supported lipid bilayers was \textit{in situ} null ellipsometry (72,73). This is an optical method where the change in polarization of light upon reflection from a substrate is monitored.

Polarized light can be divided into a parallel (p) and a perpendicular (s) component in relation to the plane of incidence. Reflection of polarized light at a surface results in a phase shift, as well as in an alteration in the amplitude, between the reflected and incident light. In ellipsometry these changes are measured in terms of the optical angles \( \psi \) and \( \Delta \), where \( \tan \psi \) describes the change in amplitude (E) ratio between the reflected (r) and incident (i) light for the parallel and perpendicular components, which can be translated into the reflection coefficient (R), and \( \Delta \) describes the corresponding phase (\( \delta \)) change between reflected and incident light:

\[
\tan \Psi = \left| \frac{E'_p}{E'_s} \right| = \left| \frac{R_p}{R_s} \right| \tag{1}
\]

\[
\Delta = \left( \delta'_p - \delta'_s \right) - \left( \delta'_i - \delta'_s \right) \tag{2}
\]

Furthermore, \( \psi \) and \( \Delta \) are related to the optical properties of the system according to the following optical model:

\[
\tan \Psi e^{i\Delta} = \frac{R_p}{R_s} = f(\lambda, \phi, n_0, n_1, n_2, d_1) \tag{3}
\]

where \( \lambda \) is the wavelength of the light, \( \Phi \) is the angle of incidence, and \( n_0 \) and \( n_2 \) is the refractive index of the bulk and the pure substrate, respectively, (Figure 4). From this model the refractive index (\( n_1 \)) as well as the thickness (\( d_1 \)) of the adsorbed layer can be obtained. From each set of \( \psi \) and \( \Delta \) two unknown parameter can be resolved. To determine the characteristics of more complex surfaces, as in Figure 5, more sets are needed and can be ob-
tained, e.g., by performing measurements in several ambient media, different wavelengths, or different angles of incidence (74).

![Figure 4](image_url)

*Figure 4. Light reflection at a thin film with a refractive index $n_1$ and thickness $d_1$ on a surface.*

From thickness and refractive index, in turn, the adsorbed amount ($\Gamma$) can be calculated by de Feijter’s formula (75):

$$\Gamma = d_1 \frac{(n_1 - n_0)}{dn/dc}$$

(4)

where $dn/dc$ is the refractive index increment.

The results obtained in the present work are primarily interpreted in terms of the adsorbed amount, since this parameter is more robust than the refractive index and layer thickness. When studying adsorbed amounts only, separation of the different layers of the substrate is not needed.

**Silica substrates**

Silica surfaces are suitable as substrates in ellipsometry since they are smooth and highly reflective (73). In order to avoid instability caused by spontaneous oxidation of silicon during measurements (76) the silicon slides were oxidized prior to use generating a SiO$_2$ layer with a thickness of 300 Å. Following oxidation the substrates were cleaned by boiling in basic peroxide solution followed by acidic peroxide solution in order to remove surface contaminations (77). Prior to use the substrates were further cleaned by plasma treatment in low pressure residual air, resulting in surfaces with a contact angle less than 10° as determined by interferometry. In addition to protecting from oxidation, presence of an oxide layer facilitates accurate determination of the thickness and refractive index of a film adsorbed on the surface (73). This, however, requires that the properties of the substrate are determined by first studying the pure substrate and its oxide layer in two ambient media (3-layer model), whereupon the adsorbed film is characterized by a 4-layer model (Figure 5).
Experimental setup

The instrumental setup is described in Figure 6. The light source, an argon laser, generates a light beam and the accurate polarization state of the light is controlled by means of polarizers and a compensator. When polarized light of a particular ellipticity hit the substrate the reflected light will become linearly polarized. After the reflection, the light will go through the analyzer and finally reach the detector. As the name implies, the basis for null ellipsometry is that a minimum of light should reach the detector, which is achieved by adjusting the positions of the polarizer and analyzer, keeping the compensator fixed at 45°, until minimum light intensity is registered by the photodetector. The substrate is positioned in a trapezoidal cuvet that allows for stirring, temperature control, and flushing with the appropriate buffer.

Bilayer formation

A number of methods can be employed for formation of supported lipid bilayers. According to the Langmuir-Blodgett (LB) technique (78), a stack of monolayers are formed on the surface by repeated submersion into a monolayer-covered solution. Thus, a bilayer is formed by two monolayer leaflets onto the hydrophilic substrate. This is, however, a rather time consuming method. Spin-coating (79), where the lipid solution is added to a spinning surface, is a much faster technique. On the other hand, the lipid
bilayer formed is less well-defined. Additionally, bilayers can be formed directly in the ellipsometer either from mixed micelle-solutions or from liposomes, two strategies that, depending on the bilayer composition, have been employed in this thesis work. Disadvantageous for these in comparison to LB is that the inner leaflet in these bilayers are more tightly attached to the surface, separated only by a thin water layer (69,70), which risks reducing lipid mobility in the inner bilayer leaflet.

Zwitterionic supported bilayers were deposited on the silica substrate from a solution of mixed micelles (69) composed of the non-ionic surfactant DDM (n-dodecyl-β-D-maltoside) and DOPC (and in paper I-III also cholesterol). After addition of mixed micellar solution adsorption was allowed to stabilize. After stabilization the cuvette was rinsed in order to remove surfactant and unadsorbed lipids. By repeating this procedure and subsequently lower the amount of the mixed micellar solution added, stable and densely packed bilayers are formed (80) as shown in Figure 7A.

Due to incomplete adsorption when using the micelle approach for formation of anionic supported bilayers, liposome adsorption was used in this case. It has been shown that formation of bilayers can be obtained by this approach, however often mixtures of bilayers and liposomes are formed on the substrate (81). In order to avoid peptide adsorption directly to the silica through possible defects in the bilayer, positively charged polylysine, was pre-adsorbed prior to lipid addition. After removal of unadsorbed polylysine liposomes were added and the adsorption allowed to stabilize as shown in Figure 7B. Similar characteristics in terms of thickness, refractive index, and adsorbed amount were obtained compared to the zwitterionic bilayers.

**Figure 7.** Formation of supported lipid bilayers (Γ (●) and d (□)) according to the mixed micelle-approach for DOPC/cholesterol (A) and by liposome adsorption for DOPC/DOPA/cholesterol (B). The amount of mixed micellar and liposome solution is given and R indicates where rinsing starts. For details, see Paper I.
Electrochemical methods

To investigate details of peptide action on phospholipid membranes electrochemical methods are well suited since these are highly sensitive to minor defects in insulating materials. In this context, the hanging mercury drop electrode (HMDE) is useful since well-defined and largely defect free lipid monolayers can be formed on a smooth surface (82,83) and peptide-induced structural changes detected.

The experimental setup (84) is based on a three-electrode cell containing the HMDE working electrode positioned in the center flanked by a reference electrode (Ag/AgCl containing 3.5 M KCl) and a counter electrode (platinum). A DOPC monolayer is formed on the surface of the mercury drop by first carefully spreading DOPC dissolved in pentane at the gas-liquid interface in the cell, followed by slowly lowering the HMDE through the monolayer. In order to avoid oxidation experiments are performed under argon in deaerated electrolyte. The DOPC monolayer can be studied, e.g., by voltammetry studies, electrochemical impedance, and permeability of electroactive ions with different size and valency.

Voltammetry studies

Monolayer phase transitions induced by an electric field were investigated by two different methods (85). In fast cyclic voltammetry (CV) the current is measured as the potential changes in the region -0.2 to -1.2 V in a forward and reverse direction at a high scan rate (40 V/s). Alternating current (ac) voltammetry, on the other hand, is performed at a considerably longer time scale (5 mV/s) and in this case the monolayer capacitance (i.e., its ability to store electric charges) is measured at decreasing potentials (-0.4 to -1.2 V), while low-amplitude ac sine-wave oscillations are added at a single frequency. Phase transitions in the DOPC monolayer give rise to reduction currents and capacitance increases that result in characteristic peaks in the CV and capacitance-potential profiles, respectively (Figure 8). Thus, at potentials between -0.4 V and -0.7 V the DOPC monolayer is oriented with the hydrophobic part towards the mercury and is completely impermeable. Increasing the negative potential results in an increasing polarity of the mercury electrode, changing its affinity for the different parts of the phospholipids as well as the electrolyte (86). At around -0.94 V a first peak appears that is due to a change in the headgroup orientation which allow permeation of electrolyte through the monolayer (87). The second peak, at around -1.0 V, is due to growth of defects in the monolayer possibly resulting in a transition to a pored bilayer (88). At potential below -1.8 V DOPC is completely displaced from the mercury droplet (87).
Changes in these characteristic peaks after peptide addition to the cell electrolyte provides information on peptide-monolayer interactions.

**Electrochemical impedance**

Impedance is a complex quantity where the real and imaginary part describes the resistance and capacitance, respectively, of the electrode system in an ac circuit (89). By measuring the impedance at a potential where the monolayer is completely impermeable (see above) as a function of frequency, information on the structure and properties of the monolayer can be obtained (90). In a characteristic impedance plot the data is transformed to the complex capacitance plane, as shown for DOPC in Figure 9.

![Impedance plot of a pure DOPC monolayer. $Y_\omega^{-1}$ is the normalized admittance, i.e., the inverse of impedance. The resistance of the pure solution (at the highest frequency) and the Zero frequency capacitance (ZFC) is also shown.](image)
From the impedance plot the zero frequency capacitance (ZFC) is obtained, which in turn is related to the dielectric constant ($\varepsilon$) of the monolayer according to the following expression (91):

$$C = \frac{\varepsilon \varepsilon_0}{d}$$  \hspace{1cm} (5)

where $C$ is the specific monolayer capacitance at zero frequency (ZFC), $\varepsilon_0$ is the dielectric constant of vacuum, and $d$ is the dielectric spacing (i.e., the monolayer thickness). Changes in the dielectric properties of the monolayer upon peptide interaction can thus be monitored.

**Permeability studies**

The properties of defects formed in the lipid monolayer can be characterized by studying the permeability of electroactive ions through the layer. An intact monolayer is impermeable to electroactive ions, and any transport to the mercury surface is a result of defects or pores in the monolayer due to peptide interaction. Electroactive ions are reduced in contact with mercury, which results in a reduction-current when changing the potential in a region that covers the redox process for the electroactive ions used. This reduction can be investigated using a pulse technique where a series of potential steps are applied while measuring the current at each step (92). By studying reduction-currents of electroactive ions with different size and valency, properties of the peptide-induced monolayer defects can be obtained.

**Liposomes**

**Preparation**

Dye-encapsulated liposomes were prepared by addition of the fluorescent dye 5(6)-carboxyfluorescein (CF) to a dry lipid film of desired lipid composition. By alternately freezing the solution in liquid nitrogen and heating to 60°C followed by multiple extrusion through 100 nm membranes unilamellar liposomes were formed. Before use untrapped CF was removed by gel filtration by running the sample through a column.

When liposomes composed of *E. coli* lipids were used freeze-thawing was replaced by stirring for one hour while heating to 50°C, due to destabilization during freeze-thawing.
Leakage studies

Peptide-induced leakage from liposomes was investigated by fluorescence spectroscopy. As described above, liposomes were encapsulated by a fluorescent dye, CF, which is self-quenching at the high concentrations used inside the liposomes. Upon peptide-induced membrane destabilization CF is released to the surrounding solution. As the CF concentration outside the liposomes is very low, no self-quenching occurs for released CF. This, in turn, results in a fluorescent signal that is directly correlated to the liposome leakage.

Size and z-potential

In order to characterize the liposomes used and also investigate whether peptides induce liposome aggregation or micellization, liposome size was determined by dynamic light scattering (DLS), also referred to as photon correlation spectroscopy (PCS). Random motions of particles, such as liposomes, gives rise to time-dependent fluctuations in light scattering. The fluctuations are dependent on the size of the particles, and by measuring the intensity autocorrelation the diffusion constant (D) of the particles can be obtained. The diffusion constant is related to the hydrodynamic radius ($R_h$) of the liposomes according to the Stokes-Einstein equation (93):

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

(6)

where $k_B$ is Boltzmann’s constant, $T$ is the temperature, and $\eta$ is the viscosity of the solvent.

Further characterization of the liposomes was performed by measuring their electrophoretic mobility, i.e., the liposome velocity when an electric field is imposed across the solution, from which the liposome z-potential can be obtained. The z-potential ($\zeta$) is defined as the potential at a position in the electrical double layer where the liquid starts to flow, referred to as the surface of shear. The electrophoretic mobility ($u$) is measured by detecting the light scattered by the moving particles from which the z-potential can be obtained by the Helmholtz-Smoluchowski equation (93):

$$\zeta = \frac{\eta}{\varepsilon \varepsilon_0} u$$

(7)

where $\eta$ is the viscosity of the solvent, $\varepsilon$ is the dielectric constant and $\varepsilon_0$ is the dielectric constant of vacuum. At the experimental conditions used the Helmholtz-Smoluchowski equation is valid for particles with a radius above
3 nm (i.e., \( R > \kappa^{-1} \)), which is the case for the liposomes used. In addition to characterizing the liposomes, z-potential measurements was used to investigate if peptides cause an alteration of the liposome z-potential upon adsorption. In addition, information on whether the mechanism of action of the peptides is associated with a membrane charge reversal is obtained.

Circular dichroism

The peptide secondary structure was investigated by circular dichroism (CD) spectroscopy. The basis for this technique is that depending on peptide orientation, differential absorption of left- and right-handed circularly polarized light by optically active chiral groups occurs. Different secondary structures give rise to characteristic CD-spectra. By studying the CD-spectra for the peptide amide bond, i.e., in the range 200-250 nm, it was found that the peptides investigated in this thesis are predominantly randomly structured, where some peptides contain a modest \( \alpha \)-helix content. Assuming that the CD signal is linearly dependent on the ratio between helix and coil, the fraction of the peptide in \( \alpha \)-helical conformation \( (X_{\alpha}) \) can be obtained from the following relation (94):

\[
X_{\alpha} = \frac{A - A_c}{A_{\alpha} - A_c}
\]

where \( A \) is the recorded CD signal at 225 nm and \( A_c \) and \( A_{\alpha} \) are the CD-signals at 225 nm for a reference peptide at 100% random coil and 100% \( \alpha \)-helix conformation, respectively. The CD signal recorded at 225 nm was used since the difference between helix and coil is most pronounced around this wavelength (95). It is important to bear in mind that the secondary structure obtained by CD measurements is an average conformation of the peptide, and by that it is not possible to find out which part of the molecule that is involved in partial helix formation.

Tryptophan fluorescence spectra

Peptide propensity to insert into the hydrophobic part of the membrane upon interaction can be estimated by studying the fluorescence spectra for tryptophan residues in the peptide. The tryptophan emission wavelength shifts in response to the polarity of its surroundings. Thus, tryptophan incorporation into a less polar environment, such as the membrane interior, results in a shift to lower wavelengths in the emission spectra, a so-called blueshift.
Although not always straightforward, a deeper penetration generally results in a more pronounced blueshift.
Results and discussion

Effect of peptide length

Consensus peptides

In order to investigate how peptide-lipid interactions are affected by peptide length alone without altering other peptide properties such as distribution of charged and hydrophobic residues, two types of consensus sequences were used where the peptide length was varied from 6 to 24 amino acids (Table 1). These repeat sequences, originally identified by Cardin and Weintraub (19), are involved in heparin-binding, but have also been shown to be antimicrobial (96). In Paper I the consensus peptides were investigated in terms of adsorption to model lipid membranes and induction of membrane rupture, as well as regarding bactericidal activity. Studies of leakage from zwitterionic (DOPC/cholesterol) and anionic (DOPC/DOPA/cholesterol) liposomes showed that in both systems the membrane destabilizing properties increased with increasing peptide length, as exemplified for the AKK-peptides and anionic liposomes in Figure 10A. This length effect was observed also in terms of peptide adsorption to supported bilayers (Figure 10B), suggesting that peptide density at the lipid bilayer is an important factor for peptide-induced membrane rupture. This length-dependent adsorption is related to the loss in translational and conformational entropy penalty upon adsorption being lower for longer peptides, as displayed by polymers, proteins, and other macromolecules (97).
To investigate whether this membrane destabilization was associated with an alteration of the bilayer electrostatic potential the liposome z-potential in the absence and presence of peptides at different concentration was measured. As shown in Figure 11, the shorter AKK6 only marginally reduced the magnitude of the liposome z-potential, in line with the marginal adsorption of this peptide, whereas AKK24 even caused a charge reversal for the anionic liposomes. This is in agreement with the length-dependent peptide adsorption observed by ellipsometry. However, since the magnitude of the liposome z-potential is higher in absence of peptides, peptide-induced liposome destabilization is not caused by a global electrostatic potential buildup inducing osmotic stress and curvature changes in the bilayer. This is consistent also with DLS findings, showing that liposome size was largely unaffected by peptide-induced leakage, thereby excluding micellization and liposome coalescence as leakage mechanisms.

**Figure 10.** A) Leakage from anionic (DOPC/DOPA/cholesterol) liposomes induced by AKK6, AKK12, and AKK24. B) Adsorption of AKK6 and AKK24 to supported anionic (DOPC/DOPA/cholesterol) bilayers investigated by ellipsometry.

**Figure 11.** Liposome z-potential for anionic liposomes in the absence (♦) and presence of AKK6 (●) and AKK24 (▲). The peptide/lipid ratios are the same as used for the leakage studies.
Electrostatic interactions play a central role in all of the systems studied in this thesis. For the consensus peptides, the effect of electrostatics has been addressed by altering the bulk properties in terms of electrolyte concentration, and also by varying bilayer as well peptide composition. For AKK24, both adsorption and liposome leakage is considerably reduced upon increasing the salt concentration (Figure 12). A similar effect was found upon replacing the positively charged arginines and lysines by histidines (AHH24:1), which are uncharged at pH 7.4. Although there is still considerable peptide adsorption to lipid bilayers, the peptide-induced liposome leakage is lost. This shows that for AKK24 non-electrostatic interactions partly promote adsorption, and that electrostatic interactions are needed for rupturing the membrane.

HKH peptides

The peptide length effect was addressed also in Paper II, but in this case peptides originating from high molecular weight kininogen (HMWK) were employed (18,98). The peptide length was altered by truncating the native peptide HKH20 at different positions into half its original length (Table 1). As can be seen in Figure 13A, reducing the peptide length eliminates the liposome destabilizing ability also in this case, as was also found in terms of antibacterial activity. The peptide adsorption, in analogy with the liposome rupture, of the shorter HKH10 is significantly lower than that of HKH20 (Figure 13B). Quantitatively, however, HKH10 adsorption is higher compared to the shortest consensus peptide investigated in Paper I. Differences in charge densities in addition to peptide length might be of importance in this case. For the consensus peptides the charge density is directly propor-
tional to the peptide length, whereas for the HKH peptides the halved peptide length is accompanied by a peptide charge reduction from +7 (HKH20) to +2 (HKH10). This means that the amount of peptide charges attached to the surface is considerably lower in case of HKH10, which in turn provides an explanation for the inability of the peptide to rupture the membrane despite the relatively high adsorption density. Increasing the electrolyte concentration almost completely eliminates both adsorption and subsequent membrane rupture for HKH20, showing that electrostatic interactions are crucial.

**Figure 13.** A) Leakage from liposomes composed of DOPC/DOPA/cholesterol induced by HKH10 and HKH20. Leakage in Tris buffer containing additional 150 mM NaCl is also shown (dotted line). B) Adsorption of HKH10 and HKH20 to supported DOPC/DOPA/cholesterol bilayers. For HKH20 adsorption in Tris buffer containing additional 150 mM NaCl is also shown (HKH20 NaCl).

**Effect of peptide composition**

**Complement peptides**

In paper III and IV the effect of peptide charge and hydrophobicity on peptide-membrane interactions was further investigated. A 21 amino acid peptide, constituting the C-terminal part of the complement peptide C3a, was used as template (CNY21, Table 1) (17). The composition was altered, as shown in Table 1, by substituting two histidines by either leucines (CNY21L), increasing the peptide hydrophobicity while keeping the net charge unchanged, or by lysines (CNY21K), thereby increasing peptide net charge. In addition, in one peptide all positive charges were removed by substituting the four arginines in CNY21 by serines (CNY21R-S). The pep-
tide net positive charge is crucial for the membrane-destabilizing properties also for this peptide, demonstrated by CNY21R-S neither adsorbing to zwitterionic or anionic bilayers nor causing any liposome leakage (Figure 14 and Figure 15). Increasing the peptide hydrophobicity compared to the native CNY21 peptide results in a modest increase in peptide adsorption (Figure 15), which is paralleled by the membrane destabilization being more efficient upon increasing the peptide hydrophobicity (Figure 14). As will be discussed in more detail in the next section, this is accompanied by a deeper penetration of CNY21L into the hydrophobic part of the membrane compared to the other peptides, thereby destabilizing the membrane. In addition, CNY21L is efficient also in higher salt concentration, whereas the other peptides are inactivated to a greater extent by presence of salt (Figure 14), demonstrating that the importance of electrostatic interaction can be reduced by introducing non-electrostatic adsorption driving forces.

*Figure 14.* Leakage from DOPC/DOPA/cholesterol-liposomes induced by CNY21, CNY21K, CNY21L, and CNY21R-S. For CNY21 and CNY21L leakage in Tris buffer with additional 150 mM NaCl is also shown (dotted line).
Figure 15. A) Isotherm for CNY21 adsorption to DOPC/DOPA/cholesterol bilayers. B) Adsorption of 1 μM CNY21, CNY21K, CNY21L, and CNY21R-S to supported bilayers composed of DOPC/cholesterol (PC) and DOPC/DOPA/cholesterol (PC/PA).

Adsorption increases with increasing peptide concentration as exemplified by the adsorption isotherm for CNY21 (Figure 15A). As shown in Figure 15B, adsorption to anionic bilayers is approximately three times higher compared to the zwitterionic analogues. However, this does not result in an increased destabilization for the anionic systems (see Paper III for details), suggesting electrostatic interactions arrest the peptide in the headgroup area, hindering the peptide of penetrating into the hydrophobic part. Taken together, the results show that the correlation between peptide adsorption density and membrane rupture is complex, but also that a high peptide interfacial density clearly is necessary for membrane rupture by these peptides.

Monolayer studies
In order to further identify the degree of peptide penetration into the membranes and analyze the types of defects formed by the CNY-peptides, studies on peptide interactions with DOPC monolayers were performed in Paper IV. Different electrochemical methods were employed to examine how peptide composition affects peptide-membrane interactions. It was found that electrostatic interactions promote peptide adsorption while additional hydrophobic interactions result in a deeper penetration into the membrane. In particular, this is evident when comparing the different profiles for the supported DOPC monolayers obtained from fast cyclic voltammetry (CV) as well as the capacitance-potential profile after addition of CNY21K and CNY21L (Figure 16). These show that the characteristic peaks in the capacitance-potential profile for DOPC (Figure 16B) are greatly depressed after addition of both CNY21K and CNY21L, whereas in the CV (Figure 16A), recording
the same capacitance peaks but at a considerably shorter time-scale, the peak depression is marginal in case of CNY21K but dramatic in case of CNY21L. In the fast CV, there is a very short time (15 ms) for the peptide to interact with the monolayer before the phase transition, and the effects observed are dominated by non-electrostatic interactions. In the ac, on the other hand, the potential is slowly decreased (80 s), and peptide adsorption driven also by electrostatic interactions can take place prior to the phase transition. Together, these findings show that for the more cationic CNY21K the interactions are largely facilitated by peptide charge, while for the more hydrophobic CNY21L monolayer interactions are due to a combination of electrostatic and non-electrostatic interactions.

Figure 16. Cyclic voltammograms (A) and Capacitance-potential curves (B) recorded 15 minutes after addition of 0.8 μM CNY21K and CNY21L (black line). The profile for pure DOPC is also included (grey line).

Furthermore, it was shown that the increased peptide hydrophobicity (CNY21L) results in a deeper penetration into the hydrophobic part of the membrane whereas increasing the peptide net positive charge (CNY21K) arrests the peptide in the headgroup region. This was illustrated by impedance measurements (Figure 17), where differences in changes in the zero frequency capacitance, related to the dielectric properties of the DOPC monolayer, were observed upon interaction with the different peptides. Thus,
for CNY21L a considerable increase of the dielectric constant was found ($\Delta \varepsilon \approx 2$), whereas there was almost no alteration upon adsorption of CNY21K ($\Delta \varepsilon \approx 0.06$).

By studying the permeability of electroactive ions of different size and valency through the Hg-supported monolayer information on pore size and structure in the membrane can be obtained. DOPC monolayers exposed to CNY21 and CNY21K allow only small amounts of Tl(I) ($R_h \approx 1.3$ Å) to pass through the monolayer (Figure 18A). Adsorption of CNY21L, on the other hand, increases the Tl(I)-permeability dramatically, indicating that this peptide causes formation of more and/or larger defects in the monolayer. These defects caused by CNY21L were further investigated by also studying permeability of Pb(II) ($R_h \approx 2.6$ Å), Cd(II) ($R_h \approx 5.3$ Å), and Eu(III) ($R_h \approx 3.6$ Å) and the results, expressed as selectivity ratio (a high value corresponding to higher permeability, see Paper IV for details), are shown in Figure 18B. As the figure shows, the monolayer is permeable also to Pb(II), but to a lower extent than to Tl(I), whereas Cd(II) and Eu(III) (the latter not included in Figure 18) only marginally pass through the monolayer. The differences observed are most likely due to the charge of the ion, since Eu(III) has a smaller hydrodynamic radius than Cd(II). However, ion size is also relevant due to the lower permeability of Cd(II) compared to Pb(II). These findings also indicate that CNY21L partly penetrate into the hydrophobic part of the monolayer and that defects most likely are formed around the adsorbed CNY21L-peptides ($\kappa^{-1} \approx 9.6$ Å). Similar defects have been shown to be induced also by magainin (61) and recently also for melittin (62).
Figure 18. A) Permeability of Tl(I) through DOPC monolayers after addition of 0.8 μM CNY21, CNY21K, and CNY21L. Also Tl(I) reduction at pure Hg, and permeability through an intact DOPC monolayer, is shown. B) Reduction of the electroactive ions Tl(I), Pb(II), and Cd(II) at a DOPC-coated Hg-electrode after addition of different CNY21L concentrations, expressed as selectivity ratio, i.e., the ratio between reduction current after peptide addition and at pure mercury at -0.6 V, where the limiting current is reached. Eu(III) is not included since its limiting current is reached at too negative potentials for it to be quantitatively compared with the other ions.

Bilayer vs monolayer

A good correlation was found between mono- and bilayers studies, in Figure 19 exemplified by permeability of Tl(I) though DOPC monolayers and leakage of CF from unilamellar DOPC liposomes, respectively (Paper IV). The finding that the influence on bilayers is similar to effects observed on monolayers show that membrane interaction of these peptides not necessarily involves the inner bilayer leaflet and that formation of transmembrane structures not are required for destabilization. The latter is compatible also with CD results, showing helix formation to be modest in the systems investigated.
Hydrophobicity increase by tryptophan end-tagging

Since studies on CNY-peptides showed that increasing the peptide hydrophobicity resulted in an increased membrane destabilization in combination with a higher salt resistance, hydrophobic peptide alterations were investigated further in Paper V, although a different strategy for the peptide design was employed. This time a highly cationic peptide, HKH10, also investigated in Paper II, was modified by tryptophan end-tags of different length (Table 1). Membranes composed of lipids more similar to those found in bacterial membranes (DOPE/DOPG and lipids extracted from *E. coli*) were used, in addition to pure DOPC membranes.

As shown in Figure 20A, tagging KNK10 by three (KNK10W3) and five (KNK10W5) tryptophan residues strongly increases peptide-induced membrane destabilization of all liposomes studied. Increasing the electrolyte concentration reduces the leakage (Figure 20B), but considerable rupture remain also at high ionic strength, particularly for KNK10W5, which is highly salt resistant. Also the antibacterial activity of KNK10W5 towards *E. coli* (Figure 20C), as investigated by Radial diffusion assay (RDA), is substantial and maintained at high salt. In addition, the cell toxicity is low confirmed by studies on both keratinocytes and erythrocytes (see Paper V for details), and also observed in a pig-skin model (99). Together, these results indicate that tryptophan end-tagging may be a promising method for generating peptides with therapeutically advantageous properties.

The membrane destabilization is again related to adsorption. Thus, at high salt concentration no adsorption of KNK10 occurs, whereas adsorption of KNK10W5 to all three membrane compositions is considerably higher than for all other peptides investigated in this thesis at this ionic strength, except for the hydrophobic complement peptide CNY21L. This show that peptide adsorption also is strongly affected by peptide hydrophobicity.
Comparison between membranes of different compositions show similar trends in terms of differences between peptides for all three membranes. However, the effect on DOPC is considerably lower in comparison to \textit{E. coli} and DOPE/DOPG, probably a consequence of the lower electrostatic potential of these membranes (-37 And -32 mV, respectively) than that of DOPC membranes (-5 mV).

\textbf{Figure 20.} Liposome leakage induced by 1 \textmu M KNK10, KNK10W3, and KNK10W5 in the absence (A) and presence (B) of 150 mM NaCl. C) Antibacterial activity of KNK10 and KNK10W5 towards \textit{E. coli} in absence (solid line) and presence (dotted line) of 150 mM NaCl determined by RDA. A high diameter (d) corresponds to high peptide-induced bacterial killing. D) Adsorption of KNK10W5 to supported bilayers in presence of 150 mM NaCl.

\textbf{Effect of secondary structure}

For many linear AMPs, peptide-membrane interaction is associated with transitions to highly $\alpha$-helical structures (4,23,100,101). However, there are also studies showing that helicity is a less important factor for membrane
disruptive activity of a number of AMPs (61,62), a finding which is becoming increasingly evident by the increasing numbers of studies performed in this area. For the peptides investigated in this thesis the helix content is low, in general, and the dominating structure is random coil both in the absence and presence of membranes (Table 2).

This demonstrates that the length-dependent effect described for the consensus and HKH-peptides above is not due to changes in secondary structure when increasing the peptide lengths. Having said that, it is worth noting that for AKK24 the helix content is almost doubled upon membrane interaction. A similar effect was observed when introducing higher salt concentrations in the buffer (Table 2). In case of the CNY-peptides the effects on secondary structure is somewhat different, where the main discrepancy occur for the more hydrophobic variant CNY21L that increases its α-helical content to 30% upon interaction with anionic membranes. However, the increased helical contents observed for AKK24 and CNY21L is not associated by increased membrane-disruptive properties for these peptide towards anionic membranes compared to the zwitterionic analogues, which implies that helix formation not is a unique prerequisite for membrane rupture in the systems investigated.

Table 2. Peptide α-helix content in Tris buffer in the absence and presence of zwitterionic (DOPC/cholesterol) and anionic (DOPC/DOPA/cholesterol) liposomes.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Tris buffer (λ)</th>
<th>+ zwitterionic liposomes (λ)</th>
<th>+ anionic liposomes (λ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKK6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>AKK12</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>AKK18</td>
<td>5</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>AKK24</td>
<td>8</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>AKK24 +NaCl¹</td>
<td>14</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>AHH21:1</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

HKH10 5 5 4
HKH20 6 8 6

CNY21 14 14 18
CNY21L 16 19 30
CNY21K 16 18 \(^2\)
CNY21R-S 10 10 11

¹ Measured in Tris buffer containing addition 150 mM NaCl
² Addition of anionic liposomes resulted in flocculation in this case as observed by DLS, thereby disqualifying quantitative analysis of the peptide secondary structure.

To summarize, these finding show that membrane rupture mechanisms based on formation of ordered peptide assemblies spanning the bilayer are less likely for all peptides investigated in this thesis.
Effect of peptide topology

In order to investigate the importance of peptide linearity on the interaction with model membranes and bacteria, comparison between a linear and cyclic version of the HKH20-peptide, investigated in Paper II, was performed (CHK22-l and CHK22-c, Table 1). Studies of this type have generally been performed for peptides where the helicity is reduced upon cyclization (102,103), which is not central here since the native peptide is in a largely random coil conformation. Cyclization was obtained by adding cysteine residues to both end terminals of HKH20 followed by ring closure. Cyclic peptides are interesting also since they are believed to be more resistant to proteolytic degradation compared to linear peptides (104).

It was found that the cyclic variant is less efficient in causing bacterial killing and liposome perturbation, related to a lower lipid membrane adsorption in comparison to the linear analogue (Figure 21). This is in line with previous findings on other cyclic peptides (102,103), and is probably related to the bulky nature of the cyclic peptide preventing peptide penetration into the membrane. It should be noted, however, that certain peptides become more potent upon cyclization (105-107), showing that the effect of cyclization is highly dependent on the peptide amino acid composition and also the mechanism of action for membrane destabilization.

![Figure 21](image)

*Figure 21. A) Leakage from liposomes composed of DOPC/DOPA/cholesterol induced by CHK22-l and CHK22-c. B) Adsorption of CHK22-l and CHK22-c to DOPC/DOPA/cholesterol bilayers.*
Correlation between model membrane studies and antibacterial and cytotoxic activity

For all peptides investigated in this thesis work, a good correlation was found between membrane destabilization and antibacterial activity towards both Gram-negative and Gram-positive bacteria, indicating that model membrane studies are relevant when exploring the mechanism of action for these peptides. This finding, as discussed in Paper I-III and V, is here exemplified for the complement peptides (CNY21, CNY21L, and CNY21K), presenting antibacterial properties similar to those of the benchmark antimicrobial peptide LL-37 (Figure 22A). Increasing the peptide hydrophobicity results in reduced sensitivity to high electrolyte concentration, observed also in terms of liposome leakage as discussed above (Figure 14). In a related study, it was shown that the CNY21-peptides also present antifungal activities (108). Comparison between model membrane studies and antibacterial investigations show that the general effects observed are similar, but detailed evaluation in terms of, e.g., concentration differences can not be made. This is expected due to the bacteria being much more complex, having components and functions not taken into account in the model membrane studies, and also due to differences in experimental design.

Figure 22. A) Bactericidal activity of the peptides against Gram-positive *B. subtilis* (grey bars) and Gram-negative *P. aeruginosa* (black bars) determined by RDA in Tris buffer in the absence (upper figure) and presence (lower figure) of 150 mM NaCl at a peptide concentration of 100 μM. B) Peptide-induced hemolysis at a peptide concentration of 60 μM, expressed as percentage of hemolysis caused by the detergent Triton X-100. The benchmark antimicrobial peptide LL-37 is also included for comparison.
The cytotoxic activity for all peptides is low, as demonstrated by the marginal hemolysis induced by the complement peptides as presented in Figure 22B. The hemolysis is actually more than three times lower for all CNY-peptides compared to the benchmark endogenous peptide LL-37. This shows that the peptides are selective towards bacterial membranes. Peptide cytotoxicity has been argued to be related to the peptide helicity (3). This relation is relevant for complement derived peptides, as it has been shown that increasing the peptide helicity by specific substitutions result in an increased hemolytic activity (109). This, in combination with liposome leakage data, suggest that secondary structure, although of marginal importance for the presently investigated systems, may contribute to the selectivity obtained for complement-based and related peptides.
Conclusions

The results presented in this thesis show that the mechanism of action of the different peptides is highly dependent on peptide composition and properties, where peptide length, charge, and hydrophobicity are of significant importance.

Clear correlations were found between peptide adsorption and resulting peptide-induced liposome leakage, although adsorption is not the only determinant for defect formation. Electrostatic interactions are crucial for promoting peptide adsorption, as observed by altering the composition of peptides and membranes, respectively, as well as bulk properties such as pH and electrolyte concentration. Peptide helix formation, on the other hand, is of minor importance for inducing membrane rupture in the presently investigated systems.

Comparative results were obtained in mono- and bilayer systems for complement-derived peptides, showing that formation of transmembrane structures are not likely, also supported by the low helix induction observed upon membrane interaction. Electrochemical experiments on DOPC monolayers for complement-derived peptides show that the peptide penetration depth into the monolayer is enhanced by hydrophobic interactions, which in turn facilitates membrane rupture. In addition, the defects formed are in close proximity of the peptide (~10 Å). Strong electrostatic interactions, on the other hand, arrest the peptide in the lipid headgroup region to a greater extent.

Hydrophobic interactions are crucial also in order to generate peptides active at higher electrolyte concentrations, of interest from a therapeutic perspective.

When comparing peptides within one group similar trends in terms of adsorption and liposome leakage were found for both anionic and zwitterionic membranes, as well as for anionic membranes of different composition, showing that as long as the membranes are stable, well-defined and contain low amounts of defects the exact model membrane composition is of less importance for this purpose. This also means that the issue of membrane selectivity is not satisfactory incorporated in the presently employed model systems.

Nevertheless, a good correlation was found between model membrane studies and antibacterial activity, investigated for both Gram-negative and Gram-positive bacteria, verifying that membrane rupture is a key mechanism...
of action for the peptides investigated. In addition, all peptides investigated are selective towards bacteria over eukaryotic membranes.
Outlook

The work included in this thesis is directed towards biophysical aspects of AMP-membrane interactions in order to further clarify the details of this interplay. For some of the peptides investigated, high potency against Gram-positive and Gram-negative bacteria is observed, whereas cell toxicity is low. Together, this indicates that peptides of the types investigated here have potential in therapeutic applications. Most promising in this context are hydrophobically modified peptides since their activities are less sensitive to physiological salt concentrations, although highly hydrophobic peptides are less selective between membranes, and have been shown to display significant cell toxicity (110,111).

There are, however, important aspects that have not been covered in the present work, namely the importance of non-lipid components in the bacterial membrane in addition to resistance development towards these AMPs. The outer surface of Gram-negative and Gram-positive bacteria is dominated by a layer of LPS and peptidoglycan, respectively. Thus, in order for AMPs to reach their main target, the bacterial membrane, they need to pass this barrier. In order to fully evaluate the importance of the membrane destabilizing properties observed, studies on AMP interaction with LPS and peptidoglycan is needed. In addition, by optimizing the AMP binding to the outer bacterial layer, membrane interaction may be further facilitated.

Resistance development is in general considered to occur to a lower extent towards AMPs in comparison to conventional antibiotics, owing to the bacterial membrane being the main target in case of AMPs. However, there are possible ways for bacteria to develop resistance also to AMPs (112,113). One important mechanism that is employed by the Gram-positive S. aureus, which is a highly important bacteria in the context of resistance, is reduction or even reversal of the bacterial surface potential, by either incorporation of D-alanine into the cell wall teichoic acid, or by L-lysine modification of PG in the membrane (114). An additional relevant resistance mechanism is production of proteases that degrade AMPs, as have been shown to occur against the human AMP LL-37 by several bacteria (115).

The main prospect for therapeutic application of AMPs lies within the field of topical formulations and other localized therapies. There are numerous skin diseases such as chronic ulcers, atopic dermatitis, and eczema where colonization of bacteria is a great problem, inducing also wound infections and infections of mucosal surfaces. By directly targeting the skin
proteolytic breakdown during systemic distribution as well as uptake difficulties are avoided. Also pulmonary delivery, for treatment of diseases such as cystic fibrosis that is associated by elevated levels of *P. aeruginosa*, is an interesting application. However, administration to the lungs may be complicated.

Finally, in order to circumvent proteolytic degradation and also reduce production costs (5), focus should be directed towards generating as short peptides as possible. In this context W-tagging (Paper V) represents a feasible strategy.


Det räcker dock inte att enbart känna till att peptiderna tar död på bakterierna genom att attackera bakterieväggarna. Utveckling av nya läkemedel kräver att man vet hur nedbrytningen sker i detalj, eftersom man då kan avgöra betydelsen av olika egenskaper hos peptiderna. För att undersöka detta används förenklade modeller av bakterier. Dessa är uppbyggda av så kallade fosfolipider, som utgör grundmaterialet i bakteriernas membran. Karaktäristiskt för fosfolipider är att de har två olika delar, en fettlös och en vattenlös. I ett membran är de fettlösa delarna riktade mot insidan av membranet, medan de vattenlösiga delarna är riktade mot omgivningen på insidan och utsidan av membranet. Olika experimentella metoder kan sedan

Är det då bara resistensutveckling hos bakterier mot substanserna som man behöver tänka på för att finna nya bakteriedödande ämnen? Nej, ett annat mycket viktigt krav på de här substanserna är att de är selektiva. Det innebär att de bara attackerar bakteriemembran och inte andra cellmembran i kroppen, eftersom det skulle ge upphov till förödande konsekvenser. Peptidernas selektivitet kan undersökas genom försök där peptidernas förmåga att bryta ner exempelvis röda blodceller studeras.


För de AMPs som har använts i denna avhandling är nedbrytning av membranet betydande för att de ska döda bakterier, och samtliga peptide uppvisar god selektivitet för bakteriemembran jämfört med cellmembran, vilket är viktigt för att eventuellt kunna utveckla läkemedel utifrån dessa ämnen.
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