THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Membrane Active Arginine and Tryptophan Rich Peptides

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: A schematic illustration of the RWR peptide binding to a lipid membrane.

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Abstract

Genetic diseases, cancer and antibiotic resistant bacterial infections are causing many people a great deal of suffering every year. Treatment and cure of these medical conditions are desirable, but also challenging for the medical industry. Membrane-active peptides (MAPs) are a group of peptides that are very promising for all these medical applications. These peptides include the cell-penetrating peptides (CPPs) which have the ability to efficiently transport DNA, proteins and other macromolecules into cells, and the antimicrobial peptides (AMPs) that can kill or inhibit the growth of bacteria, fungi and parasites. The properties of these peptides are overlapping, and have been suggested to depend on both the peptide sequence and membrane constituents. The mechanisms of action of these peptides are, however, not yet fully understood.

The goal with the work presented in this thesis, therefore is to better understand the mechanistic details of how membrane-active peptides bind to and interact with cellular membranes. The work focuses on the influence of the amino acids arginine and tryptophan on peptide function and membrane interaction, which has been investigated using cellular and physicochemical studies. The results from the work in this thesis indicate that the number and position of the amino acid tryptophan in the peptide sequence affect both cellular uptake and antibacterial properties. The biological effect seems partly related to the secondary structure of the peptides and the composition of the lipid membrane. The results also show that cellular uptake gains from increasing arginine content, and that proteoglycans at the cell surface specifically affect the cellular uptake of arginine-rich peptides. In addition, it is shown that the properties of the peptides may be affected by fluorescent labeling, and that this effect depends on the peptide amino acid sequence. In summary, these results give further information about how specific amino acids may affect the properties of the peptide, and how this regulates both membrane function and membrane specificity of membrane-active peptides.

Keywords
cell-penetrating peptides, antimicrobial peptides, membrane-active peptides, arginine, tryptophan, cellular uptake, membrane-binding, proteoglycan, cholesterol

List of Publications

I. **Effects of Tryptophan Content and Backbone Spacing on the Uptake Efficiency of Cell-Penetrating Peptides**
H.A. Rydberg, M. Matson, H.L. Åmand, E.K. Esbjörner, B. Nordén
Biochemistry 51 (2012) 5531-5539.

II. **Membrane interaction and secondary structure of *de novo* designed arginine-and tryptophan peptides with dual function**
H.A. Rydberg, N. Carlsson, B. Nordén
BBRC 427 (2012) 261-265.

III. **Peptide-membrane interactions of arginine-tryptophan peptides probed using quartz crystal microbalance with dissipation monitoring**
H.A. Rydberg, A. Kunze, N. Carlsson, N. Altgärde, S. Svedhem and B. Nordén
To be submitted to European Biophysics Journal.

IV. **Cell surface binding and uptake of arginine- and lysine-rich penetratin peptides in absence and presence of proteoglycans**
H.L. Åmand, H.A. Rydberg, L.H. Fornander, P. Lincoln, B. Nordén, E.K. Esbjörner
Biochim. Biophys. Acta-Biomembr. 1818 (2012) 2669-2678.

V. **Characterization of a novel cell penetrating peptide derived from human Oct4**
E. Harreither, H.A. Rydberg, H.L. Åmand, V. Jadhav, L. Fliedl, C. Benda, M.A. Esteban, D. Pei, N. Borth, R. Grillari-Voglauer, O. Hommerding, F. Edenhofer, B. Nordén, J. Grillari
Submitted to Cell Regeneration.

Contribution report

Paper I

Designed and performed the experiments. Performed data analysis and interpretation of results. Main author of the paper. Did not perform MATLAB analysis of binding isotherms.

Paper II

Designed and performed the experiments. Performed data analysis and interpretation of results. Main author of the paper. Did not perform the MIC measurements.

Paper III

Designed and performed the experiments. Performed data analysis and interpretation of results. Main author of the paper.

Paper IV

Took part in performing the intracellular uptake experiments. Took part in the interpretation of the results and writing the paper.

Paper V

Took part in designing the experiments on peptide cellular uptake and confocal imaging. Designed and performed the CD and LD measurements. Took part in data analysis and interpretation of the results and writing the paper.

Abbreviations

CPP	cell-penetrating peptide
AMP	antimicrobial peptide
MAP	membrane-active peptide
aa	amino acid
SUV	small unilamellar vesicle
LUV	large unilamellar vesicle
GUV	giant unilamellar vesicle
PC	phosphatidylcholine
PG	phosphatidylglycerol
PE	phosphatidylethanolamine
PS	phosphatidylserine
CD	circular dichroism
LD	linear dichroism
QCM-D	quartz crystal microbalance with dissipation monitoring

Contents

1. Introduction

Jules: You know what they call a Quarter Pounder with cheese in France?

Brett: No.

Jules: Tell 'em, Vincent.

Vincent: A Royale with cheese.

Pulp Fiction (1994) by Quentin Tarantino [1]

Things are not always what they seem at a first glance. And things can have different names, and functions, depending on the context. In the case with the hamburger at the well known hamburger restaurant, the different name in France might be related to the metric system and perhaps the wish to create a new name for the French market.

In my research, I started working with a group of six cell-penetrating peptides that also turned out to be antimicrobial. When added to mammalian cells, the peptides were able to cross the cellular membrane and efficiently enter cells, showing promise as transporters of drug molecules. When instead added to bacteria, the peptides inhibited the bacterial growth, and could possibly be used as a new type of antibiotics. Due to the different functions, the six peptides could be named either cell-penetrating or antimicrobial. Thus, depending on the context, here the type of cells to which the peptides were added, the peptides could have different functions, and names.

Due to the overlapping functions of cell-penetrating peptides (CPPs) and antimicrobial peptides (AMPs), the strong resemblance between the peptides has appeared the focus of attention. In addition to function, also the peptides' amino acid content, secondary structure and the mechanisms by which they interact with membranes are similar. Therefore are both cell-penetrating and antimicrobial peptides today often referred to as membrane-active peptides. The big challenge laying afore us is to gain increased knowledge about how these membrane-active peptides interact with lipid membranes. This may result in advances in several medical fields and help treating a wide range of diseases including cancer, multiresistant bacterial infections, cystic fibrosis and Alzheimer's disease.

CPPs were discovered as sub domains of naturally occurring proteins that have the ability to enter cells. The first CPP to be discovered was Tat, which is involved in the replication of the HIV virus. Next in turn was penetratin, a part of the Antennapedia homedomain protein, which is a transcription factor found in the fruit fly *Drosophila m.* [2]. AMPs are found in the innate immune system of most living creatures where they play a leading role in the first line

of defense against pathogens [3]. The first human AMPs were consequently named defensins [4]. Even though the CPPs were discovered over 20 years ago and the AMPs almost 30 years ago, their mechanisms of action are still not fully understood. It is known that CPPs use two main modes of cell entry: endocytosis and non-endocytotic direct penetration [5, 6]. The endocytotic pathway is considered to involve binding to proteoglycans at the cell surface [7]. AMPs instead achieve their antibacterial effect by either damaging the bacterial membrane or entering the bacterial cells where they bind to and inhibit the effect of molecules vital for bacterial survival [8]. The actions of CPPs and AMPs are all initiated by the peptide binding to the cell-membrane, followed either by peptide internalization or severe membrane damage. The effect seems to depend on the amino acid composition of the peptide in combination with the composition of the target membrane. In particular the amino acids arginine and tryptophan have been shown to be important for peptide function, affecting both cell-penetrating and antimicrobial properties.

The focus of this thesis is to gain deeper knowledge about how membrane-active peptides enter cells and interact with membranes. In Chapter 2 biological membranes are described, and in Chapter 3 a survey of arginine and tryptophan rich membrane-active peptides is presented. The methods used in this thesis, and the theory behind, are described in Chapter 4. The experimental work is based on studies of how varying amino acid composition and membrane constituents may affect peptide activity. Chapter 5 reviews the investigations of the effect of tryptophan content and backbone spacing on cellular uptake (Paper I), bacterial growth inhibition (Paper II), and the interaction with model membranes of different compositions (Paper III). In Chapter 6, the study of the effect of cell surface proteoglycans on the uptake of penetratin and its two all-arginine or all-lysine analogues PenArg and PenLys (Paper IV) is summarized. Furthermore, a new CPP, the Oct4-PTD peptide is presented and compared with penetratin (Paper V). The influence of the fluorescent label on these peptides' secondary structure and toxicity is also examined. The long-term goal of the studies presented in this thesis is to provide fundamental information allowing the design of membrane-active peptides with specific actions that can be targeted for specific cellular membranes and intracellular locations.

2. Cells, membranes and model membranes

2.1. The mammalian cell

Humans as well as all other animals (and all plants and fungi) consist of cells. Each individual human consists of billions of highly specialized and cooperating cells. Considering the yeast we use for baking, every yeast cell is instead one "individual". Yet, the difference between a human cell and a yeast cell is not as large as one might naively think, since all eukaryotic cells have the same basic construction in terms of its components. Imagine a medieval, fortified town. In the middle of the town one finds the castle, surrounded by a moat. In the castle lives the king or duke, deciding the fate of all the people in the town. In the town, amongst the small houses with straw roofs, people are living their lives doing their everyday tasks. A woman is selling vegetables at the market; another woman is baking bread in the kitchen. A man is feeding the pigs, and a dog is sniffing amongst the garbage in the alley. The whole town is surrounded by a wall. It is thick and robust, and only people with permission are let in and out. Now, when the cell is considered, in the center one finds the nucleus, surrounded by a nuclear membrane, in which the DNA is found (Figure 2.2.). The cytoplasm is crowded with organelles, transport vesicles, proteins, mRNA and other macromolecules, performing the daily life tasks. The whole cell is surrounded by an outer membrane, the cell membrane. Its main task is to keep all the cell constituents in the cellular compartments. This membrane also protects the cell from the surrounding environment and assures that nutrition and other molecules of need are transported into the cells and waste products are transported out. The cellular membrane consists of a lipid bilayer, in which membrane proteins that regulate transport and other cell functions are embedded. Attached to the membrane are also different forms of negatively charged carbohydrate polymers, including glycolipids and proteoglycans (PGs) which are anchored to the membrane by lipids or proteins respectively, typically on the extracellular side of the membrane (Figure 2.1.) [9]. PGs mediate interactions between the cell and its surroundings by regulating distribution of signaling molecules, acting as co-receptor for cell-cell signaling, and influencing cell motility and adhesion [10]. PGs are also involved in endocytosis, a collective name for several mechanisms that are used by the cell to internalize macromolecules and particles via formation of transport vesicles derived from the cell membrane.

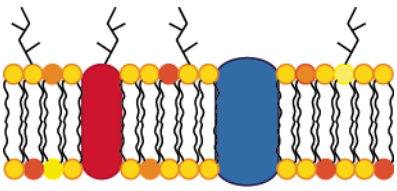

Figure 2.1. Illustration of a biological membrane, with a bilayer consisting of different lipids, embedded membrane proteins and attached carbohydrates.

2.2. Endocytosis: transport into the cell

Endocytosis is a generic name for several different processes by which cells take up material from outside the cell. Endocytosis can be divided in two main types: phagocytosis ("cell eating") and pinocytosis ("cell drinking"). The former type is only performed by specialized cells, like for example the macrophages of our immune system that can engulf pathogens and dying or dead cells. Pinocytosis is instead continuously used by most cells to ingest water and small solutes via the formation of small (around 100 nm) vesicles [9]. After ingestion from the plasma membrane, the endocytic vesicles, or endosomes, initiate their journey towards the Golgi apparatus of the cell. During this journey the endosomes "mature" from early endosomes to late endosomes, a process associated with decreased pH. After reaching the Golgi, delivering cargo, the late endosomes are converted to lysosomes, a kind of cellular "trash bins", where waste material is degraded. To make sure that the cell retains its right size, vesicles are also transported back to the plasma membrane where they fuse, a process called exocytosis.

Pinocytosis can be further divided into different types, depending on the mechanism of vesicle formation [11]. These subtypes include the clathrin mediated pathway, where coated vesicles are formed by assembly of clathrin and associated proteins, and the caveolin mediated pathway, where invaginations are formed aided by the caveolin protein and the lipid composition by the formation of so called "lipid rafts" [9, 12]. Pinocytosis also includes macropinocytosis, which differs from the other pinocytotic processes in that instead of small vesicles, membrane protrusions are formed that encapsulate and internalize both membrane-bound material and extracellular fluid [13].

2.3. The bacterial cell

One major difference between mammalian cells (and the cells of other animals, fungi and plants) is that bacteria are prokaryotes, i.e. instead of having their DNA in a special compartment (the nucleus or the "castle") as eukaryotes, their DNA is found in the cytoplasm. Bacteria are fairly simple, mostly single cellular organisms, with an enormous capacity to adapt to their surrounding environment. Bacteria are found virtually everywhere, and there are more than 50 different bacterial families [14], and very many different bacterial species. Bacteria also have a wide variety of different shapes, with the most common ones being spherical (as the *streptococcus*) or rod-shaped (as *E-coli*). Classification of bacteria is typically made according to Gram staining. Gram positive bacteria, bacteria that are stained violet by the Gram staining, have one inner lipid membrane and an outer thick peptidoglycan layer (Figure 2.2.). The peptidoglycan layer consists of a network of aminosugars, *N*-acetylglucosamine and *N*-acetylmuramic acid, that are crosslinked with peptide chains, typically including alanine, lysine and glutamine or glutamic acid. It is a thick and negatively charged layer that is very attractive for positively charged peptides and other antibiotics to bind to. The bacterial membrane surface also contains the negatively charged lipoteichoic acid, which also attracts cationic peptides [15]. Gram negative bacteria instead, have two lipid

membranes with a thin peptidoglycan layer in between. The surface of Gram negative bacteria is covered with lipopolysaccharides (LPS), a form of glycolipids. LPS have a dual role, both protecting the bacterial membrane from antibacterial molecules and also being endotoxins, as which they are toxic to the host and may cause severe inflammation and hemorrhage. Understandably, it is more difficult for drug molecules to break through the more complex membrane of Gram negative bacteria, wherefore these bacteria are generally harder to treat. [9, 16]

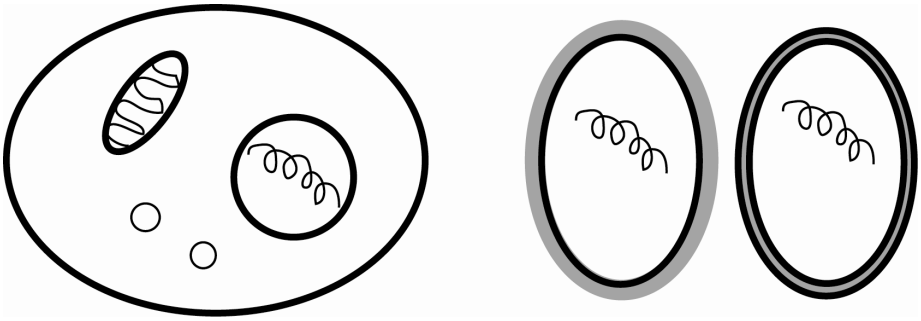

Figure 2.2. Schematic illustration, not to scale, of a mammalian cell (left) and a Gram positive (middle) and a Gram negative (right) bacterium respectively. In the mammalian cell, DNA is found in the nucleus. The Gram positive bacteria have one lipid membrane (black) and one peptidoglycan layer (gray), whereas the Gram negative bacteria have two lipid membranes with a peptidoglycan layer in between.

2.4. Biological membranes

If one considers the first cells as the origin of life, then lipid barriers or membranes have since then been used to compartmentalize enzymes, genes, and other building blocks of life from their surroundings. The ability of lipid molecules to form membranes is in fact considered a prerequisite of cellular evolution [17]. It was long believed that this compartmentalization was the only role of lipid membranes, but more and more focus has been put on their important cell regulatory functions. Indeed, the membrane is arguably one of the most complex and important organelles in the cell. Biological membranes are, at a very basic level, built up of a laterally fluid lipid bilayer with embedded proteins and attached carbohydrates. The fluidity of the membrane enables lateral diffusion of the membrane components [18], which can rearrange into different types of dynamic structures. The membrane lipids can, just like the proteins situated in the membranes, act as enzymes, signal transducers and receptors [19]. The lipid composition controls the membrane dynamics, including permeability and stiffness, and many of the cellular functions mentioned may thus be regulated by altering the lipid composition [20]. Also, the rapid change of local composition and formation of so called lipid rafts, membrane domains enriched in sphingolipids and cholesterol, might lead to the accumulation and subsequent activation of certain protein receptors [21].

The main component of the lipid bilayer of biological membranes is the phospholipids. Phospholipids consist of a hydrophilic headgroup and, typically, two hydrophobic

hydrocarbon chains commonly 16 to 18 carbon atoms long. Due to this amphiphilic nature of the phospholipids, they tend to form aggregates when in a polar solvent like water, with the hydrophilic headgroups facing the solvent. This self-assembly in water is driven by the hydrophobic effect [22]. Phospholipids have a low CMC (critical micelle concentration), meaning that already at low concentrations (around 10^{-10} M) they form complexes; and no or very few single molecules are found in solution [23].

The phospholipids of biological membranes can be divided into two major classes: phosphoglycerides and sphingolipids. They both consist of a polar phosphorylcholine head and two hydrocarbon chains, but whereas phosphoglycerides have a glycerol backbone base, sphingolipids have a sphingosine (an amino alcohol) base [24]. The properties of the lipids depend on the polar head group, but also on the hydrocarbon chain length and saturation. Depending on the size ratio between the heads and the tails, phospholipids obtain different molecular shapes. The size ratio can be described by the packing parameter, *P*, which depends on the volume occupied by the tails divided by the area per lipid molecule and the length of the tails [25-27]. If $P \sim 1$, the head and tail region are of equal size, and the shape is categorized as cylindrical. If instead the head region is bigger or smaller than the tail region, the shape becomes categorized as cone shaped ($P > 1$) or inverted cone ($P < 1$). *P* corresponds to the situation where there is no bending stress, i.e. the torque is zero, and the curvature of the lipid monolayer corresponds to the intrinsic curvature. Thus, cylindrical lipids, which have neutral curvature ($P \sim 1$), tend to form lamellar structures (bilayers) with the hydrophilic heads pointing out, and the carbon tails pointing in to the hydrophobic core region, whereas cone-shaped lipids, which have negative or positive curvature, form non-lamellar structures (Figure 2.3.) [28]. The intrinsic curvature of a monolayer can be described as the sum of the curvature of each lipid molecule [27], and the intrinsic curvature of the bilayers depends on those of the monolayers [29]. The majority of the lipids in cellular membranes have neutral curvature [30], wherefore bilayers are formed.

Figure 2.3. Schematic illustration of monolayer formation with cylindrical shaped phospholipids with neutral curvature that form lamellar structures (left) and phospholipids with negative curvature that form non-lamellar structures (right).

The energy needed to bend a bilayer from its spontaneous state, as described by Helfrich [31], is dependent on both the total curvature and the topological changes to the membrane (Gaussian curvature) [5, 32]. The curvature force per unit area of bending the membrane (*f*) is given by Equation 1:

$$f = \frac{\kappa}{2}(c_1 + c_2 - 2c_0)^2 + \kappa_G c_1 c_2 \qquad (1)$$

where c_0 is the intrinsic curvature, c_1 and c_2 are principal curvatures at a point on the surface, κ is the bending modulus (energy cost for deviation from spontaneous state) and κ_G is the Gaussian curvature modulus (energy cost of topological changes). The sum of c_1 and c_2 gives the mean curvature and the product c_1c_2 describes the Gaussian curvature. The Gaussian curvature can be used to characterize a surface. If both the principal curvatures are of the same sign, the Gaussian curvature becomes positive. Positive Gaussian curvature is found on spheres. If one of the principal curvatures is negative and the other one is positive, the Gaussian curvature becomes negative. This so called saddle shape is seen for objects with holes, like doughnuts, or membrane pores. Negative Gaussian curvature is needed for the formation of pores in the membrane, but also for the formation of invaginations and protrusions as in the endocytotic processes [5].

The diversity in lipid head groups, in combination with the many different lipid tails, results in the huge variety of lipids with different properties found in biological membranes. The combination of different lipids balances the dynamics and stability in cellular membranes, which makes the membranes both flexible and robust [33]. In addition to determining lamellar or non-lamellar phase, the lipid composition also determines in what kind of lamellar phase the lipid bilayer is, including liquid disordered lamellar phase (L_α(d)), liquid ordered bilayer phase (L_α(o)) and gel phase ($L_{\beta'}$). Phase transitions seem to be very important for membrane function, for example in the transition from the "normal" liquid disordered lamellar phase to gel phase in the formation of lipid rafts [34].

The lipid content and combination of biological membranes differ between species and between specialized cells in a species, as well as between membranes of different organelles in the same cell. In mammalian cell membranes the four most abundant lipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin. PS has a net negative charge, whereas the other three have a net neutral charge. PE is a non-lamellar lipid, with negative curvature. PE and other lipids that form non-lamellar structures are important for fundamental membrane functions. They are for example necessary for proper insertion of membrane proteins in the bilayer and for membrane protein function, as well as for fusion, fission and budding of membranes needed in endocytosis [30, 33, 35, 36]. The lipid bilayer of mammalian cells also contains 10 % to 50 % cholesterol [30], which influences the membrane rigidity and lipid rafts formation [37-39]. Bacterial membranes, in contrast, contain no cholesterol, and the content of negatively charged lipids is higher than for mammalian cells. The main negatively charged lipid in bacterial membranes is phosphatidylglycerol (PG), which, just as the negatively charged PS found in mammalian membranes, is cylindrical in its molecular shape. Gram positive bacteria generally have a high content of PG, the membrane of the Gram positive bacteria *S. aureus*, for example, contains only PG-lipids (phosphatidylglycerol and diphosphatidylglycerol). Gram negative bacteria have a slightly lower amount of PG (20 % to 40 %), but a very high content of PE. *P. aeruginosa* has a 60 % content of PE, and the content in *E. coli* is as a high as 80% [40]. As a comparison the plasma membrane of mammalian cells contains around 20% PE [30].

2.5. Model membranes

It is easy to understand that the lipid membrane is very complex. The diversity in lipid composition, proteins and carbohydrates can make it hard to evaluate the contribution of e.g. a specific amino acid for binding to a cellular membrane. To gain a better mechanistic understanding of physicochemical properties of the peptide-membrane interaction, simplified model membranes with regulated constituents are typically used. Lipid vesicles, or liposomes, are commonly used for spectroscopic assays, whereas flat supported lipid bilayers are used in combination with surface sensitive techniques (Figure 2.4.). Liposomes are categorized according to lamellarity (multi- or unilamellar) and size, typically as small vesicles of roughly 20-100 nm, large vesicles of approximately 100 nm to 1 μm and giant vesicles of >1 μm. For spectroscopic assays large unilamellar vesicles (LUVs) of around 100 nm are used, and small unilamellar vesicles (SUVs) are typically used for the formation of supported lipid bilayers.

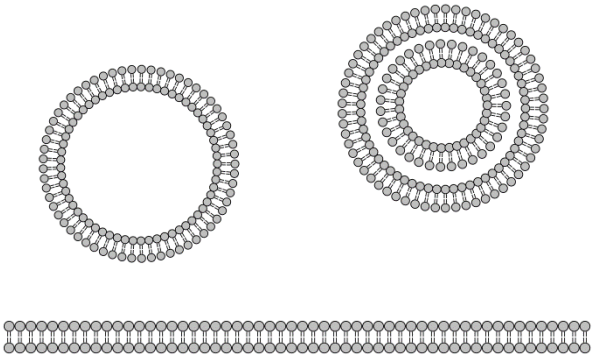

Figure 2.4. Illustration of a unilamellar lipid vesicle (left), a multilamellar lipid vesicle (right) and a flat lipid bilayer (bottom).

As main model membrane in the work presented in this thesis, bilayers consisting of 80 mole% phosphatidylcholine (PC) and 20 mole% phosphatidylglycerol (PG) were used as a rough mimic of biological cell membranes. This lipid composition is common in biophysicochemical experiments, and it is a simplified, general model of biological membranes. In addition, model membranes of PC only, and PC/PG membranes with and without the addition of cholesterol or the glycolipid Lactosyl PE have been used in the work in this thesis (Figure 2.5.) to investigate how the lipid composition may affect the peptide-membrane interaction. The negatively charged lipids of biological membranes account for a major part of the negative charges on the membrane surface. However, for healthy mammalian cells most negatively charged lipids, like PS, are located in the inner leaflet of the bilayer, while cancer cells have higher levels of negatively charged lipids in the outer leaflet. The content of negatively charged lipids in bacteria is normally high, ranging from around 20 % (*E. coli*) to being the major lipid constituent in the membrane (*S. aureus*) [40]. Membranes of biological cells also contain other negatively charged moieties, including the proteoglycans and the glycolipids, as well as the bacterial peptidoglycans and lipopolysaccharides. In cancer cells, the negatively charged carbohydrates are often

overexpressed, which contributes to the increased negative charge on the cell surface of these cells. Since the majority of CPPs and AMPs are positively charged, the negative charges of the membrane are of special interest due to their influence on the membrane interaction and subsequent cellular uptake or growth inhibition.

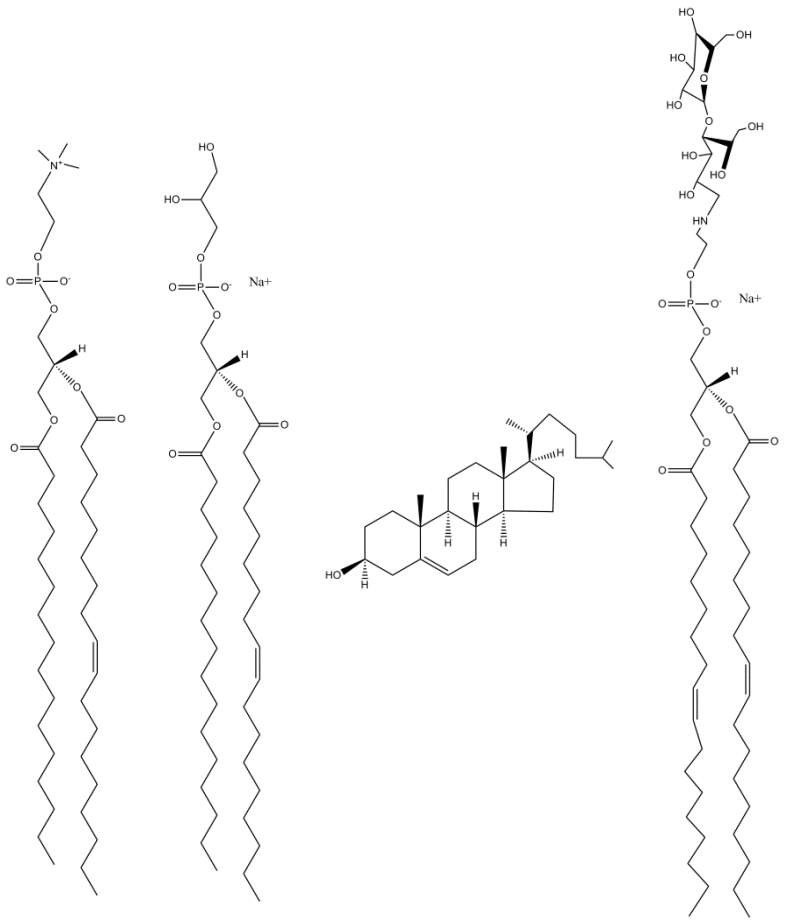

Figure 2.5. The lipids used in the work of this thesis. POPC is zwitterionic, whereas POPG is negatively charged at physiological pH. Cholesterol decreases the fluidity of the membrane and Lactosyl PE is a glycolipid with two unsaturated carbon chains and a lactose moiety.

3. Membrane-active peptides

Peptides are built up of smaller units called amino acids (aa). There are 20 different natural amino acids, which all consist of a central carbon, an amino group, a carboxyl group and the specific aa side chains. The amino acids are divided in groups depending on their chemical properties. These groups are the acidic, the basic, the uncharged polar and the non polar amino acids. In this thesis, the two amino acids arginine (basic) and tryptophan (non polar) (Figure 3.1.) will be particularly in focus, since they are commonly found in the sequences of membrane-active peptides and they are especially important for peptide function. Amino acids are linked together by so called peptide bonds: the carboxyl group of one aa residue condenses with the amino group of another one, and so on, forming longer chains (Figure 3.1.). Chains of up to 50 aa are normally referred to as peptides, whereas longer chains, are generally classified as proteins. That is, peptides are small proteins, or pieces of proteins. Depending on the combination of amino acids in the peptide or protein, the aa chain can fold into different secondary structures, including α-helixes, β-sheets and coils. [41]

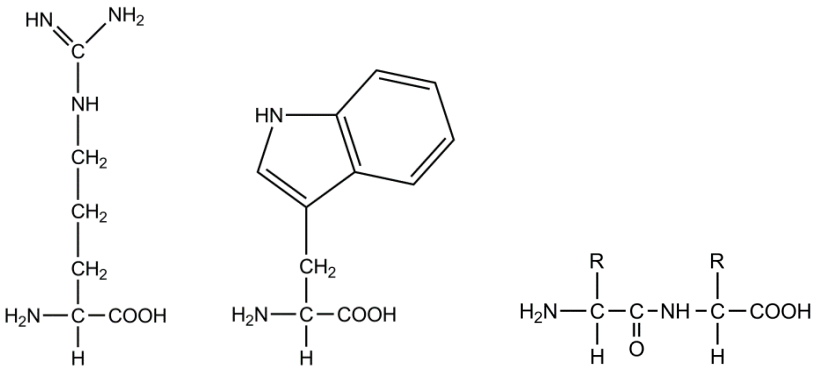

Figure 3.1. The amino acids arginine (left) and tryptophan (middle) and two amino acids (with the side chain R) linked together with a peptide bond (right).

Proteins are the most abundant group of molecules in our bodies [42]. Whereas DNA encodes the genes, proteins are the molecules in the cell that execute the instructions given by the genetic information. Mentioning just a few examples of the many functions proteins have, they catalyze chemical reactions, they act as structural components of the cell and they can be hormones as well as hormone receptors. Even though many of the proteins in our bodies are large and complex, also the smaller peptides have important functions. For example, there are peptides that act as growth factors, regulating cell growth and differentiation, and there are peptides important for the function of the nervous system [16].

Yet other peptides have important tasks in the immune system or for transport of proteins across cellular membranes. These peptides belong to a group of peptides called membrane-active peptides, which is the focus of this thesis. The membrane-active peptides include cell-penetrating peptides and antimicrobial peptides, peptides which all perform their action by interacting with cellular membranes in different ways. Cell-penetrating peptides are peptides with the ability to cross cellular membranes, transporting macromolecular cargo, whereas antimicrobial peptides inhibit the growth of bacteria and fungi. Membrane-active peptides also include fusion peptides, peptides that are able to fuse membranes for example used by viruses in their pathogenic mechanism, and sometimes also amyloid peptides, the peptides that amongst others have been associated with Alzheimer's disease. Fusion peptides and amyloid peptides will, however, not be covered further in this thesis. The actions of membrane-active peptides make them very promising for different medical applications, for example as drug transporters or as antibiotics in the case of cell-penetrating- and antimicrobial peptides, areas where new approaches are sought after.

3.1. Cell-penetrating peptides

Gene therapy is perhaps the most exciting discovery in medical therapy since the discovery of penicillin. Realization of functional gene therapy would make it possible to help millions of people and cure diseases we today do not have the possibility to cure. Just to name a few, cystic fibrosis, Huntington's disease and certain types of cancers are examples of medical conditions that could be cured with gene therapy. Gene therapy is typically based on the insertion of a DNA sequence that once inside the cell nucleus can "correct" the genetic defect by coding for the "healthy" version of the gene. Other therapeutic approaches for genetic diseases have also been tested, and include the introduction of macromolecules into the cell cytosol, for example siRNA or proteins, that interfere with transcription and subsequent biosynthesis of proteins. Unfortunately, there is still no good way to achieve this. The macromolecules proposed for therapy, mostly nucleic acids or proteins, are big, bulky and often charged, which means that they do not readily cross the cell membrane. They thus need to be transported into the cell. To date most attempts have been based on viral vectors [43], which have been shown to have several drawbacks including severe immune responses and the risk of the virus's pathologic ability being restored. A promising approach for this task is the use of cell-penetrating peptides, which show relatively high cellular uptake and low toxicity [44, 45]. CPPs are, according to the Handbook of cell-penetrating peptides, peptides of 5 to 40 aa, with the ability to efficiently enter cells, via both endocytotic and non-endocytotic mechanisms, that can bring with them macromolecular cargo like proteins or nucleic acids [46]. CPPs are typically cationic with hydrophobic motifs, often enriched in arginines and tryptophans, and they can be derived from natural proteins or be peptides designed in the lab [47]. There are also neutral and anionic cell-penetrating peptides [48], but these will not be covered further in this thesis.

The CPPs were discovered over twenty years ago when it was shown that the transcription-transactivating (Tat) protein of the HIV-1 virus could enter cells [49]. Soon after, the

Antennapedia homeodomain protein was also shown to enter cells [50]. Interestingly, both the Tat protein and the Antennapedia protein are not only specialized for cell penetration/internalization but are also transcription factors, binding to DNA and regulating gene expression. Many CPPs are indeed derived from transcription factors, and in particular homeodomains, for example the pIsl peptide [51]. Homeoproteins are, in the fruit fly *Drosophila m.*, responsible for the development of the different segments of the body (or in short that the right thing grows out at the right place). Antennapedia is one example of a homeoprotein. This protein is normally expressed in the leg segment of *Drosophila m.*, and is responsible for the growth of the legs during development. A specific mutation in the gene coding for the Antennapedia protein has been shown to cause expression of the protein in the fly head, where the antennas then are replaced by legs (pedia) [52]. This mutation has led to the name of this special protein.

During the years following the discovery of the CPPs, a great deal of effort was put into trying to find the minimal peptide sequence needed for cellular internalization. For the Tat protein this sequence was identified as Tat48-60, today commonly called Tat [53]. The minimal sequence of Antennapedia was identified as the 16 amino acids of the third α-helix of the protein [45], and was given the name penetratin. Both Tat and penetratin have a high content of arginines. Hence, a number of synthetic peptides only containing arginines were developed, which still retained the ability for cell internalization. The synthetic poly-arginine peptides have been extensively studied since then. It has for example been shown that uptake efficiency increases with the length of the poly-arginine chain [54-56] and that the spacing of the arginines in the amino acid sequence influences uptake efficiency with the arginines at every third position being optimal [55]. The optimal length of the poly-arginines has been debated, with numbers ranging from 15 [54] to several hundred arginine residues [56]. Octa-arginine (R_8) is one of the most commonly used poly-arginines, since it has high uptake efficiency into cells [57], is an efficient vector [58]; at the same time it is relatively cheap to synthesize.

The specific role of arginine in cellular uptake and membrane interaction of CPPs has been the interest of many investigations (some examples being [54, 59-64]), where one approach has been comparison with peptides rich in lysine, another positively charged, nitrogen containing amino acid. It was shown that peptides containing a high number of arginines seem to have higher uptake efficiency than lysine rich counterparts. As an example, Åmand *et al.* [65] showed that a penetratin analogue where all lysine residues were substituted by arginines had better internalization efficiency than the original penetratin (the original penetratin, with lysines and arginines, is depictured in Figure 3.2.). The higher uptake efficiency of arginine has been attributed its guanidinium group, which can form stable bidentate hydrogen bonds with anions on the cell surface [66-69], thereby generating negative Gausian membrane curvature [5]. In comparison with lysine, arginine also has a longer aa side chain, which in the case of polyarginine and polylysine, can result in both higher binding because of a larger "effective reach" and decreased toxicity because of higher charge separation and subsequently lower curvature strain on the outer membrane [70].

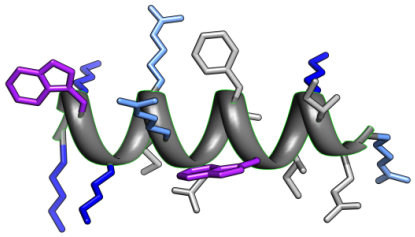

Figure 3.2. Penetratin in its α-helical structure with tryptophans in purple, arginines in light blue and lysines in dark blue. Depending on for example lipid composition and peptide to lipid ratio, penetratin can also adopt a β-sheet structure [71-75].

Another amino acid that has gained attention for influencing CPP function is the hydrophobic tryptophan. When interacting with lipid bilayers, tryptophan becomes, due to its aromaticity, positioned at the polar-apolar interface, close to the carbonyl groups of the lipid molecules [76, 77]. Tryptophan is often found in the transmembrane regions of membrane proteins where it has important anchoring functions [78-80]. Likewise is tryptophan important for the function of many cell-penetrating peptides. For example have the two tryptophan residues of penetratin been shown to be crucial for cellular uptake, where the replacement of the tryptophans with phenylalanines, and in particular the W48F substitution[1], resulted in impaired intracellular uptake [45, 81]. Tryptophan has also been shown to be vital for the insertion into the lipid membrane and cellular internalization of a group of glycosylated CPPs and their conjugated cargo [82] and the CPP Pep-1 is dependent on its tryptophan rich tail for efficient targeting to cell membranes [83]. The importance of tryptophan has been further investigated by Maiolo *et al.* [84] who showed that intracellular uptake improved significantly when adding a tryptophan at the C-terminal end of a hepta-arginine. These and other findings have resulted in investigations of CPPs consisting of only tryptophans and arginines, for example by Sagan and Alves [85, 86]. The full picture of the importance of these two amino acids for peptide function is however still not known.

3.1.1. Uptake mechanisms of CPPs: endocytosis and direct uptake

CPPs can be internalized into cells by both endocytotic pathways and non-endocytotic, direct membrane penetration mechanisms [5, 6, 87]. Direct penetration was for a while regarded to be an artifact, since the early experiments were made on fixed cells which are permeable to most macromolecules. The fixation of cells thus led to artificial results showing peptides inside the cells that were internalized *post mortem*. However, after the development of live cell assays it has repeatedly been shown that direct penetration does occur, even though endocytosis is still considered a main pathway [7, 47]. Interestingly, some sequences, in particular those rich in arginines, appear to be able to use both routes in parallel [54, 60, 88].

The first step of internalization of CPPs is the electrostatic interaction between the peptides and negatively charged moieties on the cell surface. The interaction with proteoglycans plays

[1] W substituted for F in position 48

a major role in this context, and can activate different routes of internalization, including macropinocytosis [89] and clathrin- and caveolin-dependent endocytosis [88, 90] (Figure 3.3.). For arginine-rich peptides a specific dependence on the interaction between arginines and proteoglycans for cellular uptake has even been suggested [91]. Proteoglycans are basically long chains of glycosaminoglycans covalently attached to a core protein, which can be attached to the lipid bilayer of the cellmembrane. Proteoglycans are believed to be important for cell signaling, for example by binding signal molecules and enhancing their effect by locally increasing their concentration. It has been proposed that they may act in the same way regarding arginine-rich CPPs [92]. Since the peptide concentration affects which uptake routes are utilized, increased concentration of peptide at the cell surface may probably be a part of the uptake mechanism, and accumulation of arginine-rich peptides on the surface may lead to the induction of macropinocytosis. The initiation of macropinocytosis could for example be triggered by proteoglycan aggregation or receptor activation by the CPPs cross linking receptors [92], even though CPPs are not considered to be dependent on any specific receptor for uptake. Endocytosis is described further in Section 2.2.

The initial electrostatic interaction between the peptides and negatively charged moieties on the cell surface may also lead to direct, non-endocytotic, ATP-independent, translocation through the membrane (Figure 3.3.). Translocation has been attributed to several different mechanisms, including pore formation (barrel stave- and toroidal model), formation of inverted micelles, the carpet-model and membrane thinning. These structural rearrangements are however often associated with membrane leakage. Other mechanisms, like the formation of bleb-like structures, flip flop of the lipids and the formation of a porous structure caused by the Gaussian curvature induced by arginine binding have also been suggested [92], as well as clustering of anionic lipids. All mechanisms are dependent on factors that compensate for the energy needed to transport the charged CPP through the lipid membrane. The transmembrane potential is suggested to be a driving force in the translocation of peptides, confirmed by studies using liposomes [93] and simulations [94]. There are also theories about cationic peptides being able to form neutral complexes with anions, which would facilitate the transport through the membrane [92]. Peptide-membrane interactions are further described in Section 3.4.

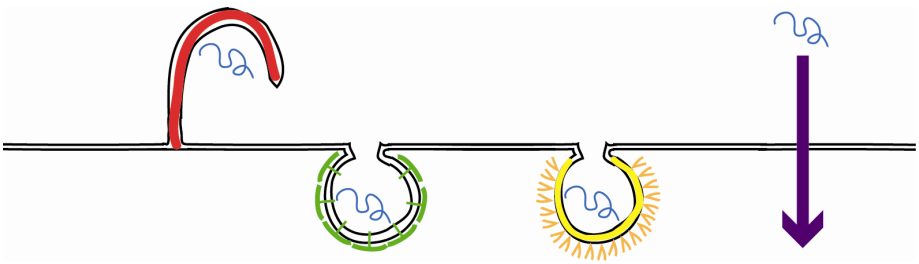

Figure 3.3. Cell-penetrating peptides (blue) are internalized into cells by several different routes, including macropinocytosis (red), clathrin-mediated endocytosis (green), caveolin-mediated endocytosis (yellow) and direct translocation through the cellmembrane (purple).

The entry mechanisms, as well as the final intracellular destination, seem to be dependent on several factors, like the nature of the attached cargo, cell type and even cell cultural conditions [57, 95]. The amino acid sequence and subsequent secondary structure also play a major role for CPP uptake. A more pronounced, often α-helical, secondary structure has been associated with increased uptake, but contradicting results have also been obtained showing no such connection [56, 96-98]. The concentration of the CPP has also been proven important, and as an example, Ziegler *et al.* [99] showed that, for the peptide WR_9, endocytosis was the predominant uptake route at low peptide concentration whereas direct penetration occurred at higher concentrations. The subject is complex. The fact that the same peptide can use several different routes in parallel, and that the inhibition of one route often leads to activation of other routes, makes it difficult to study CPP uptake mechanisms and to compare the results from different studies. This makes it intriguing to learn more about the uptake mechanisms and in particular about the peptide-membrane interactions.

3.1.2. CPPs in the medical industry

The typically low toxicity of CPPs makes them promising for the medical industry [44]. During the last decade, the number of clinical trials as well as companies working with CPP-based medical techniques has constantly increased [47]. It therefore seems possible that we in a relatively near future will be using CPPs as therapeutics.

Already in the early days of the CPP, experiments showed that the cell-penetrating peptides could carry macromolecular cargo with them. The cargo could be virtually any biomolecule, including proteins, peptides, nanoparticles, DNA and siRNA [46, 47]. The cargo can be covalently attached to the CPP or just mixed with the CPP (non-covalent strategy) [47]. The latter was first reported by Morris *et al*. who showed that the peptides MPG [100] and Pep-1 [83] could deliver cargo using a non-covalent approach. CPPs function as drug vectors both *in vitro* and *in vivo* [101]. Ever since Schwarze *et al*. reported that Tat could be used for protein transduction in mice in 1999 [102], the *in vivo* use of CPPs has grown, almost flourished. There are extensive reports on the matter (for a review see for example [7, 46, 47, 103]), which make CPPs promising also for future treatment of diseases in humans. Cell-penetrating peptides have a true ace up their sleeves: they can cross the blood brain barrier, which normally is impermeable to most drug molecules due to the tight junctions between the endothelial cells in the cerebral microvascular endothelium. CPPs can thus be used to transport drugs into the brain, as exemplified by the fusion protein of Tat and the Bcl-xL protein, that efficiently entered mouse brain where it decreased neuronal cell death [104], but may also be used for transport of nano-molecules for visualization of brain tumors in patients [103]. CPPs have also been used as delivery molecules in the treatment of asthma and other inflammatory diseases [46].

Cancer therapy is one big area of focus for CPP utilization. Cancer, extremely briefly, is caused by cells with abnormal control mechanisms of growth and proliferation. One important molecule involved in these processes is the tumor suppressor protein p53. Normal cells can sense when something is “wrong”, like in the case of metabolic disorder or genetic damage. When p53 receives information about such faults, it will tell the cell to stop growing and fix

the problem. In the worst case, if the fault is not fixable, p53 will initiate apoptosis, the programmed cell death. In cancer cells, control mechanisms like this are “turned off”, exemplified by p53 being found malfunctioning in many types of cancer [105]. Therefore, one way to treat cancer is to “turn on” these control mechanisms. Tat has for example been used for efficient delivery of a p53 reactivating peptide [106]. There are also many other examples of successful use of CPPs in cancer therapy, targeting p53 as well as other growth controlling proteins in the cell [46, 47, 107]. CPPs have also been used in radioimmunotherapy for transport of antibodies conjugated with radioactive isotopes to tumor cells, and to enhance the effect of conventionally used chemotherapeutics [103].

Importantly, CPPs (and AMPs) have been shown to have selective interaction with cancer cells which make them, if possible, even more interesting for cancer therapy [108-110]. Cationic CPPs are known to preferentially bind to negatively charged membranes, and cancer cells have more negatively charged membranes than normal cells. This difference in charge is due to the increased anionic charge on the surface of cancer cells as a result of over expression of certain proteoglycans and glycolipids, as well as an increase of the negatively charged lipid phosphatidylserine in the outer leaflet of the membrane. Some CPPs in fact appear to have anti-tumor activity, while being non-toxic to the healthy cells, possibly due to this cancer cell selectivity. Peptides with anti-cancer activity have similarly been found to also function as CPPs [111].

3.2. Antimicrobial peptides

The spread of resistant bacteria, leading to untreatable infections, is a major public health threat and one of the big challenges in modern medicine. The overuse, or even abuse, of antibiotics is one cause to this threat. People often, rightly, blame the meat industry or the over prescription of antibiotics by physicians. Often forgotten in this context is the use of antibiotics in soap and washing powder and the use of antibiotic substances in shoes and sport clothes to prevent “unpleasant” odours, which can really be considered unnecessary use. Another dangerous source to resistance is antibiotic producing factories in for example India, where antibiotics are leaking out into the surroundings, where appallingly many variations of bacteria resistant to multiple antibiotics can be found. Bacteria are tough guys that fast can adapt to new situations. The high number of individual bacteria in a colony in combination with fast growth, make them survival experts and they can rapidly develop resistance to drugs. The resistance gene can then easily be transferred to other individuals, both of the same and of other species, causing escalating resistance to antibiotics.

A promising approach in fighting bacteria is the use of antimicrobial peptides. These peptides have a key role in the innate immune system, where they take part in the first line of defense against pathogens like bacteria, fungi and enveloped viruses. In humans, AMPs are found on epithelial surfaces including the skin, the gastrointestinal tract and the respiratory tract [112], where they are synthesized by epithelial cells both continuously and upon pathologic activation. AMPs can also be released from cells of the adaptive immune system. Released

AMPs have many immunoregulatory functions and can for example act as chemokines and attract (other) cells of the adaptive immune system to the infection site and neutralize bacterial endotoxins [113-116]. AMPs also function by killing the pathogen, either by damaging the cell membrane by, for example, forming pores, or by crossing the cell membrane and binding to intracellular targets and thereby inhibiting the metabolism of the pathogen cell. In this thesis, the AMPs action against bacteria will be in focus.

The existence of proteins with antibacterial activity has been known since the early 1960 [117]. Around 20 years later the first antimicrobial peptides were identified. AMPs were first discovered in plants (for example [118]), then in moths [119], followed by the first mammalian AMP in 1983 [120]. Two years later the first human AMPs, called defensins, were discovered by Ganz *et al*. [4]. The amount of discovered AMPs has increased constantly since, and to date, August 2013, more than 2200 different AMPs have been identified [121]. Amongst these can be mentioned, the magainins found in the skin of Xenopus in 1987 [122], the tryptophan- and arginine rich peptide indolicidin [123], and the membranolytic peptide melittin [124, 125] which has been used in the work of this thesis as a positive control of membrane disruption.

AMPs are in average around 30 aa long, but peptides of down to six aa have also been found to be efficient bacterial inhibitors [121]. By use of combinatorial libraries of synthetic peptides, the hexapeptide Ac-RRWWRF-NH2 was identified as effective antibacterial agent [126]. The peptide sequence has been improved by Dathe *et al*. who amongst others have made cyclic versions of the peptide [127]. The fact that an arginine- and tryptophan rich peptide was found to be the most promising antibacterial candidate is not surprising. Arginines and tryptophans are common amino acids in AMPs, as in CPPs, [121] and the content of these amino acids has been proven important for peptide function. The antibacterial effect increases with increasing arginine content [128] and with the insertion of one or several tryptophan moieties into peptide sequence [62, 129, 130]. The specific importance of arginines and tryptophans for antimicrobial peptide function has for example been demonstrated by Vogel *et al.* [131]. Arginine has, as mentioned above, the ability to form bidentate hydrogen bonds with phosphate head groups and other negatively charged moieties of the cell membrane, which affect both the degree of binding and toxicity. Tryptophan also has interesting properties. Because of its indole side chain, tryptophan is considered to be hydrophobic. Using Wimley-White's hydrophobicity scale, tryptophan is even *the* most hydrophobic amino acid [132]. However, the π-electron system of the tryptophan indole, i.e. its delocalized electron clouds, can participate in cation-π interactions. Cation-π interactions are important for substrate-enzyme binding, but they have also been suggested to be vital in arginine-tryptophan interactions since they contribute to a more energetically favorable entry of arginines into the lipid bilayer [133]. Tryptophans may also interact with the lipid choline group [134] and they have been suggested to interact specifically with glycosaminoglycans on the cell surface [135]. The role of arginines and tryptophans in peptide function is indeed exciting, and has also been explored further by the development of synthetic arginine and tryptophan peptide mimics [136-138].

In addition to specific amino acids, the secondary structure of the AMPs can also affect their antibacterial function [8]. Structure-function analyses are often made, and have revealed a correlation between α-helical structure and antibacterial effect [139]. The secondary structure is an often used criterion for division of AMPs into subgroups. AMPs (as well as CPPs) are generally disordered in solution and adopt an ordered secondary structure upon binding to the membrane, but there are exceptions. The different classes of secondary structures include the α-helical peptides, the β-sheet peptides and the extended peptides [8, 140, 141].

The α-helical peptides, which is the largest group [121], are disordered in solution but form a secondary amphiphatic helical structure upon membrane interaction. The formed helix normally has the hydrophobic aa residues at one side and the charged aa residues at the opposite side of the molecule. The antibacterial effect of these peptides depends on the extent of the formed hydrophobic surface [8]. The magainin peptides belong to this family. If the peptides are long enough they may create membrane spanning pores [141], but they can also use other membrane disturbing mechanisms like the formation of toroidal pores [8] (Figure 3.4.). Depending on the charge and hydrophobicity of the amino acids in their sequence, the α-helical peptides can be more or less deeply inserted into the membrane, which also affects their toxicity [142].

The β-sheet forming peptides are stabilized by disulfide bridges, and have their secondary, often ball-like, structure both in solution and when bound to membranes. The disulfide bridges are essential for the formation of the secondary structure, but their importance for the antibacterial activity of the peptides is still unclear. There are reports stating that these bonds are vital for the peptide action but other works demonstrate that they have no effect at all [143-145]. The defensins have β-sheet structure. The β-sheet peptides can form toroidal pores in membranes and there are even examples of β-barrels formed from peptide oligomers [146] (Figure 3.4.).

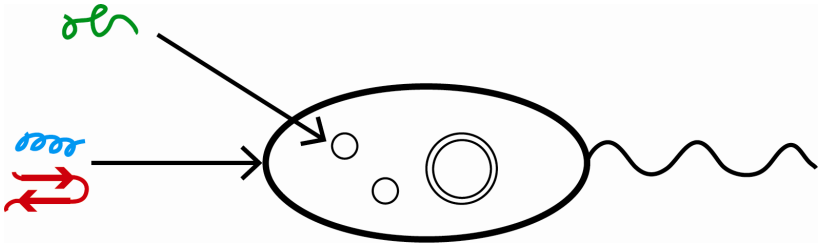

Figure 3.4. AMPs use several different mechanisms to inhibit bacterial growth. The extended AMPs (green) often act by inhibiting intracellular targets, but they may also disturb the membrane. The α-helical (blue) and β-sheet forming peptides (red) generally act by membrane disturbing mechanisms.

The extended AMPs do not fold into any specific secondary structure. These peptides are often found to inhibit bacterial growth by crossing the membrane [131] where they inhibit key cellular processes like the synthesis of proteins and nucleic acids [113, 147-149] (Figure 3.4.). Arginine and tryptophan rich AMPs, e.g. indolicidin, often belong to this group [8, 150]. In

addition to the inhibition of intracellular targets [148], indolicidin and other extended peptides can act by several mechanisms including anion transport across the membrane [151], as well as membrane perturbation [152]. The different models of how AMPs act on membranes, will be covered more thoroughly in Section 3.4.

3.2.1. AMPs in the medical industry

As for CPPs, AMPs, in addition to bacteria, show activity against cancer cells, as well as selectivity for the negatively charged membranes of these cells [70, 153-156]. This makes AMPs promising both as a new type of antibiotic but also for cancer treatment. AMPs can also be used to enhance the effect of commonly used antibiotics [157]. Furthermore, the enhancement of the expression of endogenous AMPs using gene therapy has also been suggested [158]. This could help patients with cystic fibrosis who have decreased antimicrobial peptide function in their airway mucosa [159]. There are, however, some obstacles that need to be overcome before the AMPs can reach the clinic. There are issues regarding the cost of manufacturing these peptides, their predisposition to proteolytic degradation and the possible toxicity when systemically administered. One way to reduce the cost of production is to use shorter, peptide analogues instead of native AMPs, like the peptide library derived hexapeptides. Shorter peptides are, however, even more sensitive to degradation. The peptide stability can be improved by PEGylation [160], by the use of D-amino acids and by making cyclic peptides [161]. The introduction of arginine mimetics in peptide sequences has also been shown to increase the peptide stability [162]. There are already some peptide based antibiotics in the clinic, but so far, only for topical use [163]. A combination of polymyxin B (a lipopeptide) and gramicidin S (a cyclodecapeptide) is used in the clinic for topical use [157] and the indolicidin derivative omiganan has shown promising results in prevention of catheter infections and acne in phase III clinical trials [3, 164]. There are currently also AMPs for systemic use both in preclinical and phase III clinical studies [114]; it thus seems like the wide use of AMPs as antibiotics is under way.

Nevertheless, are there concerned thoughts about the possible development of peptide resistance in bacteria (reviewed in [165, 166]). In particular the use of human derived AMPs has been considered risky, since it could potentially lead to the emergence of bacteria that are resistant to the AMPs of our own immune system, which could seriously compromise our first line of defense against pathogens [167]. The development of bacteria resistant to AMPs is considered unlikely, since the action of AMPs generally is faster than the bacterial growth rate (the bacteria are killed before they have a chance to use evolution to create resistance). Furthermore, AMPs' major target is the cellmembrane and it would be too costly for the bacterial cell to change its membrane composition in order to prevent AMP induced damage, and a change in composition could also be lethal for the bacteria [153]. Likewise, is the development of specific proteases for degradation of cytosolic AMPs considered to be too costly. In addition, most multicellular organisms have a wide array of different AMPs that they can use when attacking bacteria, so the risk of resistance to one of them is considered unlikely and negligible [153, 157]. Even if the risk of bacterial resistance to AMPs is already small, the risk could be further reduced by using improved peptide sequences with increased efficiency and membrane specificity.

3.3. CPP or AMP or MAP?

CPPs and AMPs have historically been divided according to their membrane action. CPPs are non-toxic peptides that are internalized into cells via endocytosis and/or direct translocation. AMPs are peptides that act by damaging cell membranes. Yet it is not that simple. With the discovery that some antimicrobial peptides do not damage the membrane but rather act by binding to intracellular targets, in addition to the reports about the strong resemblance between CPPs and AMPs [168, 169], the view about what AMPs and CPPs are needed revision.

CPPs and AMPs are in general short, cationic, amphiphilic peptides, rich in arginine and tryptophan. All the mechanisms of action of these peptides include interaction with cellular membranes. Comparing the CPPs and the AMPs, it is clear that they are very similar. In fact, CPPs and AMPs are so similar that several CPPs, including penetratin [128, 170], pVEC [170, 171], TP10 [171] and Tat [172], have been shown to have antimicrobial properties, and several AMPs, such as Magainin [173], Lactoferrin [174] and LL-37 [175] have been shown to be cell-penetrating. Moreover, short synthetic peptides rich in arginines and tryptophans have been proven effective as both transporters and antibacterial agents [84, 126, 176]. The similarity between CPPs and AMPs makes it plausible to think that the mechanisms of membrane interaction are similar. The action of membrane-active peptides is initiated by the electrostatic interaction between the cationic peptide and anionic moieties of the membrane. Then, some peptides damage the membrane and some are internalized. Shaw *et al*. show that the different functions of different peptides, being AMP or CPP, can be coupled to their different interactions with lipid membranes [177]. The peptides induce different changes to the membrane upon binding, an effect which is also dependent on the peptide concentration. The peptide function, being either cell-penetrating or bactericidal, seems to depend on the lipid composition of the target plasma membrane [178, 179] and on the peptide sequence [180-182]. Thus, the same peptide can have different action when interacting with different membranes due to the composition of the target membrane.

The strong resemblance and the overlapping functions of CPPs and AMPs make the use of the term “membrane-active peptide” convenient as a generic name for all peptides that perform their action by interacting with membranes. This name is, however, sometimes considered to be applicable only for peptides that induce evident membrane disruption [8]. In this thesis, I use the term “membrane-active peptide” for all cell-penetrating peptides and antimicrobial peptides, independent of their molecular mechanism of action, since all their actions start with the binding and further interaction with cellular membranes. Still, there are peptides with specific actions, either as CPP or AMP. Furthermore, the potent membrane damaging peptides, like melittin, are not likely to be useful as CPPs. However, increasingly often peptides are found to have multiple actions and there are constantly new such peptides discovered. For example the p14ARF peptide, a peptide that mimics the function of the whole p14ARFprotein involved in cancer progression, has also CPP properties [111]. The sequence resembles that of known CPPs, with high content of arginines. Thus, the amino acid content

in combination with the membrane composition might determine if the peptide is a CPP, an AMP, a MAP, or even an anti-tumor peptide.

3.4. Membrane interactions of arginine and tryptophan rich peptides

The binding of a membrane-active peptide to a biological membrane is generally initiated by an electrostatic interaction between the cationic peptide and anionic moieties of the membrane. This is followed by folding/compaction of the peptide, and then either perturbation of the membrane or translocation of the peptide through the membrane. The interaction can be seen as interplay between the peptide and the membrane, with changes in peptide secondary structure and induced changes to the membrane. In theory, the electrostatic interaction would, because of screening and the high dielectric constant of water, not be very prominent for peptide binding in an aqueous environment. However, the release of condensed counterions on the membrane surface leads to a large entropic gain upon peptide binding, which in turn leads to a strong electrostatic interaction. The peptide sequences of CPPs and AMPs include both charged and hydrophobic amino acids residues. The peptides are generally amphiphilic or form secondary amphiphilic structures when binding to membranes. For example, the peptide CADY forms an α-helical secondary amphiphatic structure upon binding [183]. For the rearrangement of the peptide secondary structure upon membrane binding, both electrostatic and hydrophobic interactions are needed [184]. Whereas a high net charge makes the peptide bind to the lipid head region, the hydrophobic interaction is considered important for deeper insertion of the peptide into the membrane [185]. When the peptide binds to the membrane, the peptide may change its secondary structure, but also the membrane will change its conformation and it normally tends to bend around the peptide. Even though there is a loss of energy due to this deformation of the membrane, it is energetically favorable for the membrane to wrap around the peptide, since this causes maximum contact, maximum counterion release and thus maximum entropy gain [5].

The mechanisms of membrane deformation caused by peptide binding include the formation of stable or transient pores and changes in the lipid packing order or loss of material via detergent like mechanisms [8, 70, 153]. All the mechanisms seem to have in common that the peptides first bind horizontally at the surface, and when a certain (local) concentration is reached, pores or other alterations of the membrane integrity are induced (Figure 3.5.). In the barrel-stave pore model, transmembrane pores are formed by the peptides forming a “barrel” in the membrane of amphiphilic α-helices with their hydrophobic part facing the hydrophobic interior of the lipid bilayer. Toroidal pores are caused by the induction of a positive curvature strain on the membrane which results in a pore forming, or an opening of the membrane, where the edges are covered with lipids. The carpet model refers to when the peptides after binding at high concentration at the surface cause membrane permeation and disruption, for example by causing micelle formation in a detergent like way. The toroidal pore formation can also lead to micelle formation upon increased peptide concentration. Moreover, loss of

lipid material, as well as lateral expansion of the membrane caused by peptide binding, can result in membrane thinning as the tails of the phospholipids relax.

Peptide binding can also result in lipid segregation and domain formation, by the attraction of negatively charged lipids or the lipid with the lowest phase transition temperature to the positively charged peptides, which may compromise the membrane stability and cause leakage [177, 179, 186-188]. Alterations in the lipid packing may also result in conformation changes of membrane proteins and thus impaired membrane function [153] or membrane protein activation. Furthermore, some peptides, for example those rich in arginine, can induce lipid membrane phase transitions [5]. Arginine can, due to its guanidinium group, induce formation of negative Gaussian curvature. When peptides that can induce negative Gaussian curvature bind to membranes containing cone-shaped negative intrinsic curvature lipids, or non-lamellar phase forming lipids, phase transition into cubic phase may occur. The negative intrinsic curvature lipids include for example PE. Also cholesterol, only found in eukaryotic cell membranes, enhances ability to form negative Gaussian curvature [5]. In addition is negative Gaussian curvature associated with pore formation and with endocytosis where both the formation of vesicles and the formations of protrusions in macropinocytosis are dependent on the membrane curvature. Negative Gaussian membrane curvature has even been associated with the formation of macropinocytotic-like protrusions in lipid vesicles with encapsulated actin [189]. The formation of negative Gaussian membrane curvature also lowers the free energy barrier for entry of the peptide through the membrane, facilitating peptide translocation.

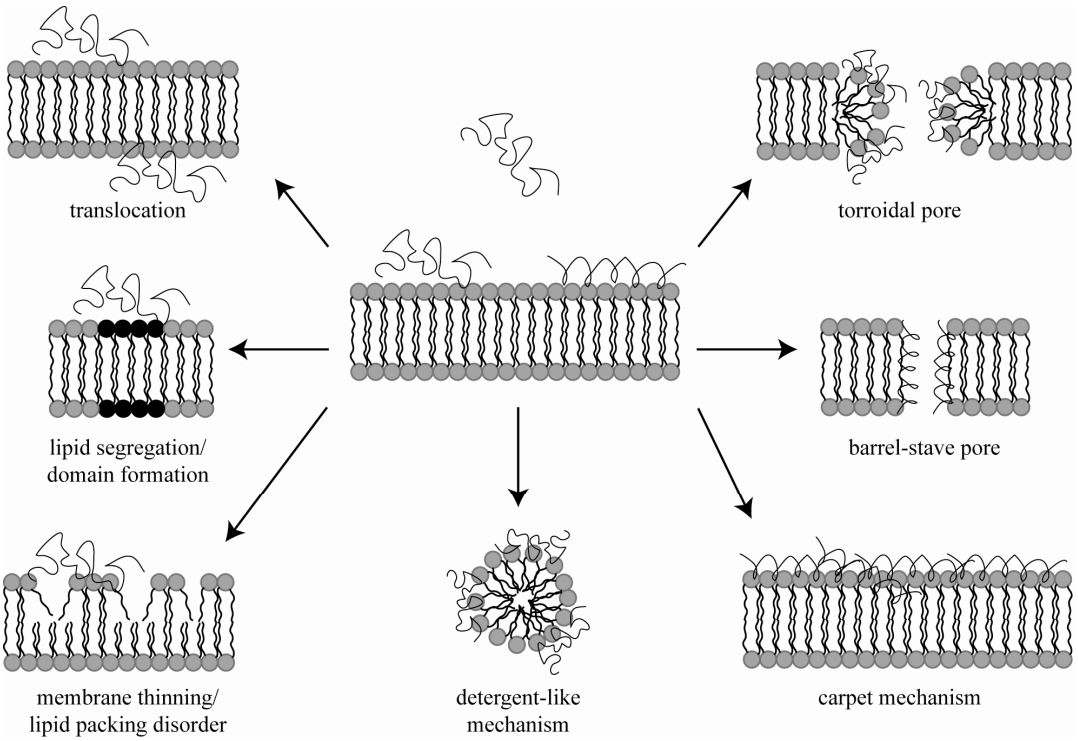

Figure 3.5. Schematic illustration of different mechanisms and end-products involved in peptide-membrane interaction leading to peptide uptake or membrane destabilization.

Translocation is caused by chemical potential imbalance induced by the peptide adsorption to the surface, but is also a result of the driving force of the natural membrane potential of cellular membranes. Peptide adsorption to the membrane may result in depolarization, seen for magainin for example [190]. This leads to irreversible membrane damage [153], or flip flop of the lipids between the outer and inner leaflets [191]. Translocation has also been associated with the formation of transient pores. It has been suggested that at a certain peptide concentration pores are formed, which aid the passage of peptides through the membrane. When more and more peptide is internalized, the concentration at the surface decreases and the pore vanishes. This could be a mechanism both for CPP internalization as well as killing of gram negative bacteria since the actual killing is believed to be caused by damage to the inner membrane [153]. The peptides may also be internalized by binding to carbohydrates and proteins of the membrane as described previously for CPPs.

Looking into the membrane interactions of CPPs and AMPs, it may be suggested that the difference in activity between the peptide classes is due to the peptide sequence in combination with the target membrane and its carbohydrate, protein and lipid composition. For example, pore formation is facilitated in PE-rich membranes, and higher content of charged and hydrophobic amino acids in the peptide sequence generally increases membrane destabilization [192]. Comparing CPPs and AMPs and combining the knowledge from the two fields, may have a synergistic effect leading to deeper understanding about membrane-active peptides and the difference between their mechanistic effects.

4. Methodology

In this chapter a brief description of the experimental techniques used for the work in this thesis is presented. A more detailed description of the methods and the experimental setups can be found in the material and methods sections of the amended papers.

4.1. Absorption spectroscopy

Light, or electromagnetic radiation, can be described as a wave, consisting of an electric and a magnetic field that oscillate perpendicular to each other and to the direction of propagation. The polarization of the electromagnetic radiation is described by the orientation of the wave's electric field. The electric field of the light emitted from a normal light bulb, or the sun, is non polarized, oscillating in a variety of directions. If the electric field instead is oriented in one plane, with a single direction, the light is described as linearly polarized. If the electric field rotates with the light wave propagation, the light is described as circularly polarized. Circularly polarized light is produced by combining two orthogonal linearly polarized light beams of equal magnitude with a phase difference of π/2. Electromagnetic radiation can also be described as discrete energy units, called photons. When light interacts with molecules, and the molecules absorb light, it is in the form of photons. Thus, when exposed to electromagnetic radiation, a molecule can absorb a photon and make a transition to a higher energy level, i.e. the molecule becomes excited. For such a transition to occur, the energy of the light must correspond to the energy difference between the final, E_f, and the initial state, E_i, described by the Bohr frequency condition (Equation 2).

$$\Delta E = E_f - E_i = h\nu = \frac{hc_0}{\lambda} \qquad (2)$$

Here, h is the Planck's constant, c_0 is the speed of light and the energy of the light is related to the frequency, ν, or inversely to the wavelength, λ. The molecule can also scatter the photon and it can emit a photon. The absorption of light is coupled to a shift in the electrical density of the absorbing molecule. The direction of this charge displacement is called the transition polarization, which together with the intensity are described as the electric dipole transition moment. The probability of a molecule absorbing a photon is the highest when the transition polarization and the light polarization are parallel. [193, 194]

Absorbance spectroscopy is one of the most commonly used techniques in bio-physical-chemical research. A schematic illustration of a spectrophotometric setup is shown in Figure 4.1. By measuring the incident (I_0) and transmitted (I) light intensities, the absorbance (A), can be determined using the Beer-Lambert law (Equation 3) [194], where l is the sample path length, c is the concentration of the absorbing sample and ε is the molar absorption coefficient, which depends on the wavelength of the light, λ.

$$A(\lambda) = log\frac{I_0}{I} = \varepsilon(\lambda)cl \qquad (3)$$

Due to the proportionality between absorbance and concentration as described by the Beer-Lambert law, absorbance spectroscopy is commonly used to measure the concentration of samples. In the work presented in this thesis, absorption measurements have, for example, been used to determine the peptide concentration and the concentration of biomass when investigating bacterial growth inhibition.

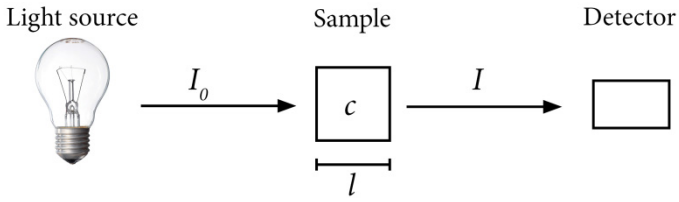

Figure 4.1. Absorption spectroscopy setup, consisting of light source, sample and detector. I_0 is the incident light intensity, I is the transmitted light intensity, l is the sample path length, and c is the concentration of the absorbing sample.

4.2. Spectroscopy of peptides

Peptides, and proteins, absorb photons in the UV region. In the region between 250 nm and 300 nm, the aromatic amino acids tryptophan, tyrosine and phenylalanine as well as disulphide bonds absorb, whereas peptide backbone and some amino acid side chains (including arginine) absorb in the region below 250 nm [194]. The transition moments of peptide secondary structures also absorb light in the UV region, with for example the transition moment of the long axis of an α-helix absorbing at 210 nm and the short axis at 190 nm and 222 nm (Figure 4.2.). In this thesis, a major part of the work has been focused on the aromatic, nonpolar amino acid tryptophan. The indole chromophore of tryptophan has three main electronic transitions, L_a, L_b and B_b (Figure 4.2.). L_a has a broad absorption at 270 nm, L_b has two absorption peaks around 290 nm and B_b absorbs at 225 nm [193]. Tryptophan has the highest absorption intensity of the aromatic amino acids, characterized by the highest molar absorption coefficient of 5690 cm^{-1} M^{-1} at 280 nm. This means that the contribution of the tryptophan absorption to the overall absorption in the aromatic region is extensive, and hence, the tryptophan absorption can be used to determine the concentration of tryptophan containing peptides. The emission of tryptophan is accordingly greater than of the other amino acids. The tryptophan emission is also sensitive to the microenvironment. This makes it possible, in addition to concentration determinations, to use tryptophan emission as a tool to study conformational changes and peptide-membrane interactions. When a peptide or protein containing tryptophans interacts with a membrane, the tryptophans normally incorporate themselves just beneath the lipid head groups. The change in local environment,

from buffer solution to the less polar lipid membrane, changes the tryptophan emission, seen by a decrease in the emission wavelength and increase in the quantum yield [195]. This change in emission was used in this thesis to determine binding constants of the peptides binding to liposomes in solution.

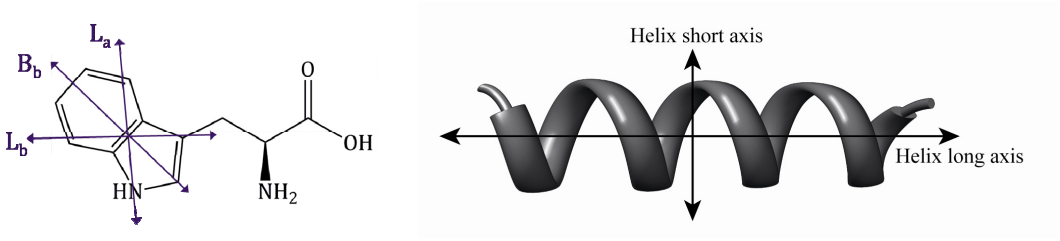

Figure 4.2. Transition moments in the indole chromophore of tryptophan (left) and in an α-helix (right).

4.3. Circular dichroism spectroscopy

Circular dichroism, *CD*, is the difference in absorption of left and right circularly polarized light passing through a sample as described by Equation 4 [193].

$$CD = A_l - A_r \qquad (4)$$

Upon absorption, the electric field of light induces a linear charge displacement, represented by the electric dipole transition moment. Also the magnetic field can induce charge displacement in molecules, but normally to a much less extent compared to the electrical field. The magnetic field, however, may induce a circular rearrangement of the electron density, represented by a magnetic dipole transition moment. In chiral molecules a combination of the electric and magnetic transition moments may create a net helical electron movement. If this is right-handed in one enantiomer, it will be left-handed in the opposite enantiomer, causing the CD to have opposite signs for the two enantiomers. For achiral molecules the net electron displacement is instead planar. Such molecules are inactive in CD since they absorb the right-handed and left-handed forms of circularly polarized light equally, cancelling them out. CD can thus be used to determine secondary structures and degree of folding of chiral molecules, like DNA and proteins. In this work CD spectra were measured for peptides, both in solution and bound to lipid vesicles, to explore their secondary structure.

4.4. Linear dichroism spectroscopy

Linear dichroism, *LD*, is the differential absorption of linearly polarized light parallel, $A_{\parallel}$, and perpendicular, $A_{\perp}$, to a macroscopic orientation axis of a sample as described in Equation 5 [193].

$$LD = A_{\parallel} - A_{\perp} \tag{5}$$

LD can be used to measure the orientation of molecules relative to a macroscopic orientation axis. In the work in this thesis, LD has been used to measure the orientation of peptides relative to a lipid membrane. In order to obtain a LD signal, the macroscopic orientation axis needs to be aligned. In this work, this was achieved using a Couette cell, which consists of two cylinders with a narrow gap in between where the sample solution is added (Figure 4.3.). Rotation of one of the cylinders creates a laminar shear flow which deforms and aligns the liposomes, with the longest dimension preferentially in the flow direction. Sucrose is added to the sample buffer for suppression of light scattering of the liposomes and matching of the refractive index, which makes it possible to measure LD signals arising from transition moments in the UV region [196].

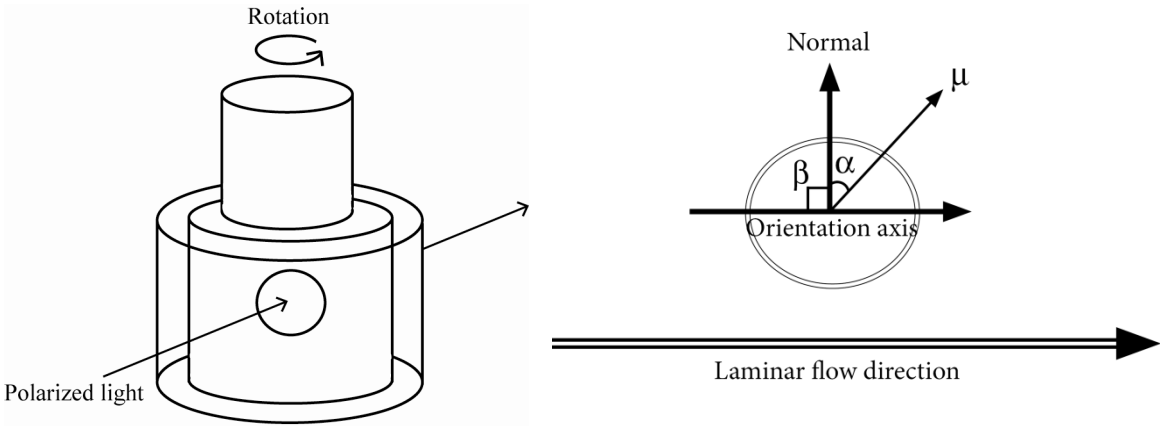

Figure 4.3. To the left, a schematic representation of the Couette cell used for the LD measurements. To the right, schematics of a liposome oriented in a laminar shear flow. The macroscopic orientation factor (*S*) is the difference between the orientation axis and the flow direction. The microscopic orientation (β) is the angle between the normal and the orientation axis, and α is the angle between the transition moment of the peptide (μ) and the normal.

From the measured LD spectra, the orientation of the peptides relative the lipid surface can be determined. A positive LD signal means that the peptides are more parallel than perpendicular to the orientation axis whereas a negative LD means that the peptides are more perpendicular. A quantitative measurement can be achieved by normalizing the LD with the absorption of

non-oriented, or isotropic, sample, A_{iso}. This value is concentration- and path length-independent, and referred to as Reduced linear dichroism, LD^r (Equation 6).

$$LD^r = \frac{LD}{A_{iso}} = 3S\frac{(3cos^2\alpha - 1)}{2}\frac{(3cos\,^2\beta - 1)}{2} \qquad (6)$$

Here, S is the macroscopic orientation factor, which varies between 0 and 1, with 1 being a perfectly oriented sample, α is the angle between the transition moment of the molecule and the normal to the orientation axis and β is a measure of the microscopic orientation (Figure 4.4.). When measuring LD of liposomes, β is 90°, thus Equation 6 is converted to Equation 7.

$$LD^r = \frac{3}{4}S(1 - 3cos^2\alpha) \qquad (7)$$

4.5. Light scattering

A molecule that is exposed to light can also scatter the light. Depending on size, shape, uniformity, etc. of the sample, more or less of the light is scattered. When light interacts with the electron cloud of a particle, an oscillating dipole moment proportional to the magnitude and direction of the incoming light, is developed. In this process a photon is absorbed, and can then be radiated again. If the radiated photon has the same frequency as the frequency of the incoming light, i.e. with the same energy as the incoming photon, the light scattering process is called elastic. If instead the radiated photon has different energy than the incoming one, the light scattering is referred to as inelastic. Scattering of light by particles that have much smaller diameter than the wavelength of the incoming light is called Rayleigh scattering [197]. For Rayleigh scattering, the intensity of the scattered light is proportional to the molar mass of the particle, the inversed fourth power of the wavelength and the scattering angle. For larger or more asymmetrical particles, the Rayleigh connections need to be modified accordingly. Light scattering is used in many measurement techniques, for example in flow cytometry (see below), and in dynamic light scattering (DLS). In DLS, light scattering fluctuations are used to determine particle size. Very briefly, due to Brownian motion, particles in the solution move into or out of the small illuminated volume element causing variations in the scattered light. The diffusion coefficient can be calculated from the scattering variations, using an autocorrelation function that is fitted to an exponential decay function. From the diffusion coefficient, the hydrodynamic radius of the particle can then be calculated. [198]

4.6. Fluorescence spectroscopy

A molecule that absorbs a photon moves from a ground state to an electronically excited state. From the excited state the molecule returns to its energetic ground state S_0. This can be achieved by a number of different pathways (Figure 4.4.), which can be divided into radiative, i.e. emission of photons, and non-radiative where the energy is transferred to the solvent environment as vibration, rotation or translation. An excited molecule that has absorbed sufficiently enough energy to end up in the second singlet excited state S_2, will subsequently lower its energy by internal conversion and vibrational relaxation in order to return to the lowest singlet excited state, S_1. From here the molecule can return to S_0 directly (fluorescence) or via inter system crossing to the excited triplet state followed by emission of a photon (phosphorescence). A third alternative route also exists, which is intersystem crossing and vibrational relaxation. The emission life time, τ, is the average time the molecule remains in the excited state and is dependent on the efficiency of the deactivation pathways which all occur in parallel. The fraction of molecules that relax to S_0 via fluorescence is described by the fluorescence quantum yield Φ (Equation 8). [199]

$$\emptyset = \frac{k_f}{k_f + \sum k_{nr}} \qquad (8)$$

Here k_f is the rate constant for fluorescence emission and Σk_{nr} is the sum of the rate constants for the other decays. The fluorescence intensity can decrease because of different quenching processes, including collisional quenching, static quenching or formation of non-fluorescent complexes, and attenuation of the incident light caused by the fluorophore itself [199]. In the work in this thesis, self-quenching of the fluorophore carboxyfluorescein has been used to investigate peptide-membrane interactions. Fluorescein is believed to self-quench as a result of the formation of non-fluorescent fluorescein dimers in combination with energy transfer from fluorescein monomers to the non-fluorescent dimers when the distance between them is equal or less than the critical Förster distance, which is about 55 Å for carboxyfluorescein [200, 201]. By measuring fluorescein emission and self-quenching using fluorescence spectroscopy, binding of peptides to lipid membranes and peptide-induced leakage of vesicles were monitored. For the binding experiments, solutions of carboxyfluorescein-labeled peptides were titrated with increasing amount of liposomes, revealing the peptide to lipid ratio at which the peptide coverage per cm^2 of the lipid surface was the highest. The peptide induced leakage of membranes was monitored by adding unlabeled peptide to liposomes containing carboxyfluorescein at concentrations high enough for self-quenching. If leakage is induced upon peptide addition, carboxyfluorescein molecules leak out into the surroundings and a fluorescence signal equivalent to the amount of leakage can be monitored.

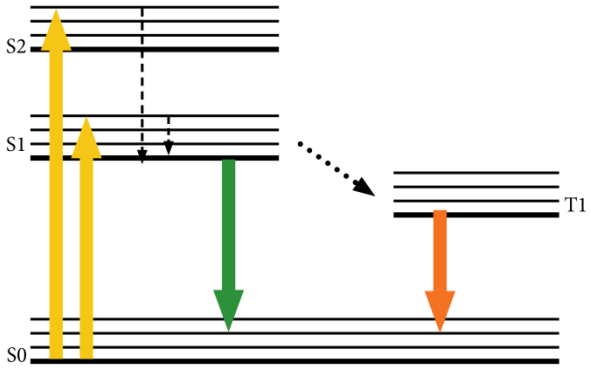

Figure 4.4. Jablonski diagram showing excitation levels and possible excitation and relaxation pathways. S_0 is the ground state, S_1 and S_2 are first and second excited states and T_1 is the first triplet excited state. Coloured arrows represent the radiative transitions absorption (yellow), fluorescence (green) and phosphorescence (orange). The non-radiative transitions are represented by dashed arrow for internal conversion and dotted arrow for intersystem crossing.

4.7. Confocal laser scanning microscopy

Confocal laser scanning microscopy was used in this thesis to image uptake and intracellular localization of fluorescently labeled peptides in live cells. Since single cells are too small (mammalian cells are around 10 µm in diameter) to be seen by the naked eye, the magnification brought by the use of a microscope is needed to visualize the cells and the peptide uptake. The resolution of the magnification depends on the wavelength and on the numerical aperture of the objective, i.e. how much light that can be collected. Confocal imaging, which gives images of high resolution and contrast, uses optical imaging to create a virtual "slice" of the specimen. In conventional microscopy, the whole specimen is exposed to the illuminating light and scattered light from areas out of focus is also detected, which reduces the image quality. In confocal laser scanning microscopy in contrast, the illuminating light is focused to a very small (diffraction limited) spot using a pinhole. Also the detector (typically a photomultiplier tube, PMT) of a confocal laser scanning microscope is equipped with a pinhole, which blocks the light that is out of focus. This renders a high spatial resolution which makes it possible to e.g. discriminate between peptides located at the cellular membrane and in the cytoplasm or the cell nucleus. In addition, the use of confocal laser scanning microscopy can reduce both photobleaching of the sample and phototoxicity to cells due to this constrained point of illumination in the xy- and z-planes. 2D images, or "slices", of the specimen are obtained by scanning in a raster pattern (line by line in a specific order) over the xy-plane. By scanning several planes in the z-direction, 3D images can be constructed. Using multiple laser sources, it is possible to monitor several fluorophores simultaneously, for example peptides labeled with the carboxyfluorescein 5-FAM (excitation at 488 nm and emission between 500-550 nm) and the marker for dead cells, 7-AAD (excitation 543 nm and emission between 600-700 nm), as in the work in this thesis. [202, 203]

4.8. Flow cytometry

In flow cytometry, light scattering and fluorescence spectroscopy are combined to monitor size, granularity and the intensity of chosen fluorescent markers of single cells in suspension. By the use of flow cytometry when measuring peptide uptake in cells, information about fluorescence intensity of each and every cell in the population of thousands of cells is monitored, instead as for bulk measurements where the average of all cells in a population is measured. In addition, statistics of the whole population is gained. In this thesis, a Guava easyCyte HT8 system was used to monitor the amount of internalized peptide in live mammalian cells. In the Guava system, the sheath flow used in conventional flow cytometry is replaced by the use of a thin 0.1 mm capillary, which renders a flow where only one cell at a time passes through the probe volume and the laser beam (Figure 4.5.).

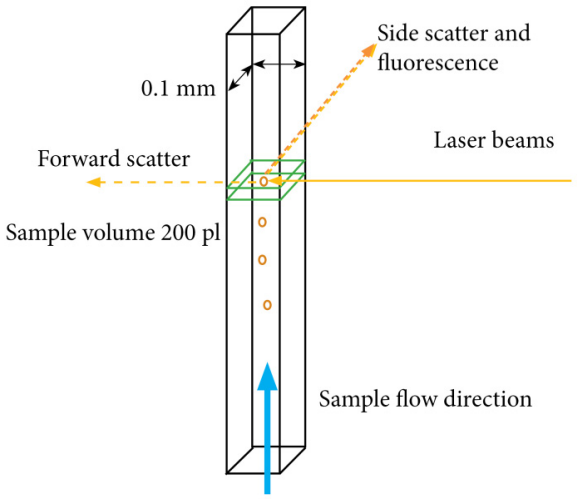

Figure 4.5. Schematics of the Guava flow cytometry setup, with the thin squared capillary rendering the controlled flow needed to measure size, granularity and fluorescence intensity of only one cell at a time. Forward scatter, or cell size, is measured in line with the laser beams, whereas side scatter, or granularity, and fluorescence intensity are measured at an angle of 90°.

Very briefly, a cell suspension is sucked through the thin capillary, creating the flow where only one cell at a time passes the laser beams. For each, side scatter (SSC), forward scatter (FSC) and fluorescence intensity is measured. SSC is referred to as the granularity signal. The more granular, or irregular, the cell is, both on the surface and inside, the more it will scatter the light. The SSC detector is placed at an angle of 90° compared to the incoming light. FSC, or the size signal, is instead measured in line with the illuminating light beam. The FSC detector detects light that is scattered, or "bent", at very small angles from the incoming light [204]. Depending on the size and volume of the cell, as well as the refractive index, the illuminating light will be bent to different extent, with more bending of light for larger cells. At the same time as size and granularity are measured, information about intracellular uptake of fluorescently labeled peptides can be gained (Figure 4.6.). The fluorescence emission is

detected at right angles compared to the illuminating light (which is conventional also in "normal" fluorescence spectroscopy). The Guava used in the work in this thesis is equipped with two lasers, one 488 nm and one 543 nm, and, in addition to the side scatter and forward scatter detectors, it has six fluorescence detectors enabling measurement of a vast variety of different fluorophores where of two different simultaneously. For example carboxyfluorescein labeled peptide and the marker for dead cells 7-AAD can be monitored at the same time, or 7-AAD in combination with Annexin-V PE (Annexin-V conjugated with Phycoerythrin) can be used to distinguish between cells in late or early apoptosis respectively. Also by analyzing size and granularity, side scatter and forward scatter can be used to distinguish live cells from dead cells and cell debris, making it possible to exclude non vivant cells from the analysis so that the results of peptide uptake reflect that of live cells only. [204]

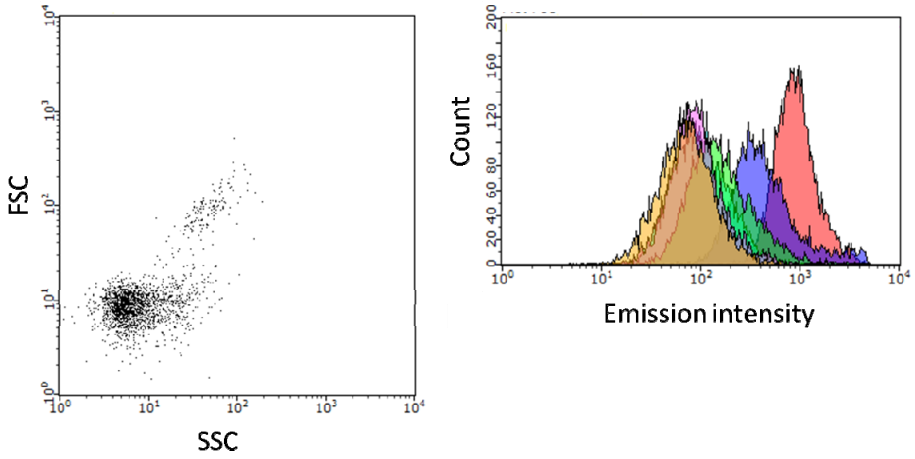

Figure 4.6. Typical read outs from flow cytometric measurements in the form of dot plots (left) and histograms (right), with the measurements based on light scattering (FSC and SSC) of one cell population and fluorescence emission of multiple cell populations respectively.

4.9. Quartz crystal microbalance with dissipation monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a surface sensitive technique that measures changes in mass/thickness or viscoelastic properties of thin layers of material on a sensor surface. The sensitivity is high and mass depositions of ng/cm^2 can be detected [205]. The technique is based on the piezoelectric properties of a quartz crystal [206]. If the crystal is subjected to an electric potential, the applied voltage can induce mechanical deformation of the crystal. This is exploited in QCM-D, where gold electrodes are deposited on both sides of an AT-cut quartz crystal disk, which oscillates in so called *shear-thickness mode* when an alternating potential is applied. AT-cut means that the crystal is cut at the angle of 35.25° from its optical axis. Resonance will occur when the frequency of the applied potential corresponds to the fundamental frequency and/or overtones of the quartz crystal sensor. Sensors with fundamental frequency of 5 MHz are normally used in QCM-D measurements (Figure 4.7.). In general, when mass is adsorbed on the sensor surface, the

oscillating frequency decreases (i.e. for thin and rigid films). In QCM-D, the alternating potential driving the oscillatory motion of the crystal is continuously switched on and off. When switched off, it is possible to measure the oscillation decay giving the damping of the system or the dissipation. The dissipation, *D*, is described by Equation 9, where $E_{dissipated}$ is the loss of energy during one oscillation and E_{stored} is the stored energy [207].

$$D = \frac{1}{2\pi} \times \frac{E_{dissipated}}{E_{stored}} \quad (9)$$

The magnitudes of the recorded frequency shifts and the associated dissipations depend on the viscoelastic properties of the material at the sensor surface, with very rigid materials having low dissipation (relative the frequency shift) and loose and soft materials having high dissipation. For materials with low dissipation, the deposited mass can be calculated from the frequency shift using the Sauerbrey equation [208] (Equation 10),

$$\Delta m = -C_{QCM} \frac{\Delta f_n}{n} \quad (10)$$

where Δm is the adsorbed mass on the surface, C_{QCM} is the mass sensitivity constant (17.7 ng cm^{-2} Hz^{-1} for the 5 MHz quartz sensors) and Δf_{n} is the change in the resonance frequency at the n$^{\mathrm{th}}$ overtone. Since the QCM-D sensor is based on the oscillation of the quartz crystal, not only the dry mass adsorbed at the surface, but also solvent molecules associated with the layer on the sensor surface are measured. For example, almost 50% of the mass deposition of peptides binding to lipid bilayers is water [49]. Hence, with QCM-D it is possible to measure highly hydrated systems, which can be difficult with other techniques, and it is for example possible to follow the formation of lipid bilayers by rupture of liposomes (Figure 4.7.). When forming supported lipid bilayers on the QCM-D sensor surface, liposomes are first adsorbed to the surface. The liposome adsorption is characterized by a large decrease in frequency and a large increase in dissipation, following the mass deposition of the soft liposomes. The rupture of the liposomes, including release of the interior content of the liposomes, is followed by the formation of the bilayer. This step in the bilayer formation is visualized by an increase in frequency (i.e. loss of mass) and decrease in dissipation (a more rigid material is formed on the surface). For a supported lipid bilayer of good quality, the shift in frequency of the final bilayer is typically between -25 Hz to -27 Hz and the shift in dissipation is typically less than $0.5 \cdot 10^{-6}$ [209].

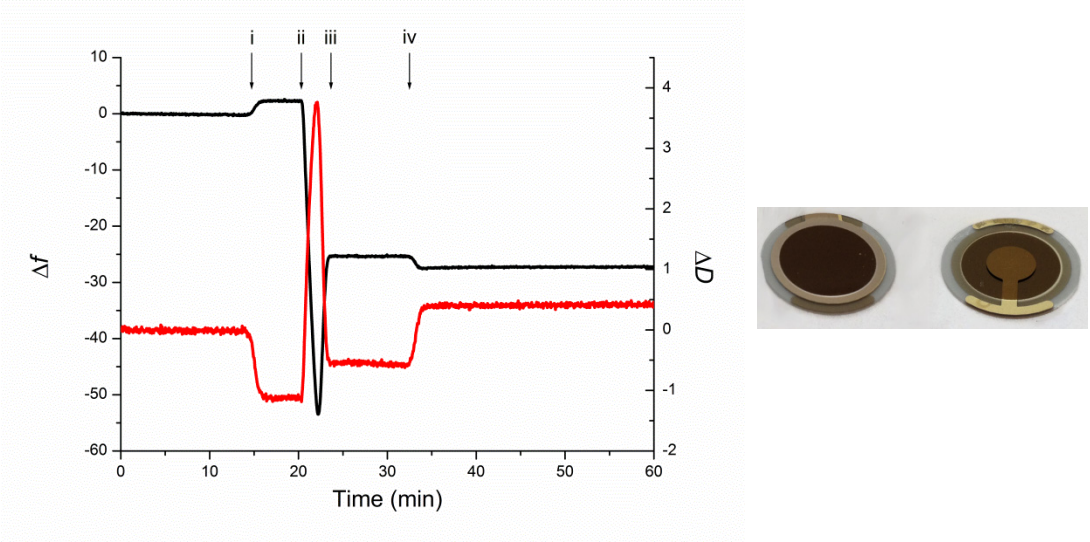

Figure 4.7. Graph of QCM-D bilayer formation (left) and picture of the two sides of the QCM-D sensor (right). In the graph, a typical formation of a POPC/POPG bilayer is shown, where i) is change of buffer from PBS to PBS supplemented with Mg^{2+}, ii) is addition of liposomes diluted in PBS Mg^{2+} , iii) is addition of PBS Mg^{2+}, and iv) is the change from PBS Mg^{2+} to PBS. The frequency signal is depicted in black and the dissipation signal in red.

4.10. Preparation of model lipid membranes

For the preparation of the model lipid membranes used in this thesis, the lipids to be used are first dissolved in chloroform. Lipid films of desired lipid composition are then prepared in round-bottomed flasks, followed by evaporation of the chloroform. Evaporation can be achieved using either a gentle stream of N_2 or a rotary evaporator. To remove any residual solvent, the lipid film is then put under vacuum for at least 2 h. Liposomes are formed by vortexing the lipid film with appropriate buffer, followed by extrusion 21 times through polycarbonate membranes with pore diameter of 30 nm (for SUVs used to form supported lipid bilayers) or 100 nm (for LUVs used in spectroscopic experiments). As an additional step to ensure unilamellarity of the liposomes, the liposomes can in between vortexing and extrusion, be subjected to five cycles of freezing and thawing in N_2 (l) and 50 °C respectively. The size of the liposomes can be measured by dynamic light scattering (DLS) or other appropriate methods, such as Nanoparticle tracking analysis (NTA). For formation of supported lipid bilayers, the liposomes are adsorbed to the sensor surface where they rupture and form a bilayer. The rupture and subsequent bilayer formation can be aided by osmosis, using different buffers inside and outside of the liposomes, or by using peptides that can break and/or fuse the lipid membranes.

5. *De novo* designed arginine and tryptophan peptides with dual action

In Paper I, II and III, the influence of tryptophan number and position on peptide function and interaction with membranes is investigated. A series of peptides, all containing eight arginines and one, two, three or four tryptophans was designed. The tryptophan residues were placed either at the N-terminus, in the middle or evenly distributed in the sequence (Table 5.1.). In Paper I, the cell-penetrating properties of these peptides are studied, including their uptake in live mammalian cells, intracellular localization and toxicity. In Paper II, the secondary structure of the peptides is examined and it is tested if the peptides may also act as antimicrobial peptides. In Paper III, the induced changes to lipid membranes of different composition upon peptide binding are investigated using surface sensitive techniques. A summary and comments to the results are presented. More thorough descriptions of methods and results are found in the amended papers.

Table 5.1. Amino acid sequences of the arginine-tryptophan peptides.

Peptide	Sequence*
WR_8	WRRRRRRRR
W_2R_8	WWRRRRRRR
W_3R_8	WWWRRRRRRR
W_4R_8	WWWWRRRRRRR
RWR	RRRRWWWWRRRR
RWmix	RWRRWRRWRRWR

*Tryptophans are shown in pink

5.1. Uptake of the RW-peptides in live mammalian cells

In order to investigate whether an increase in tryptophan content and a change in tryptophan backbone spacing might influence uptake and intracellular localization of cell-penetrating peptides, uptake efficiency of the arginine-tryptophan peptides (RW-peptides) in live CHO-K1 (Chinese hamster ovarian cells) was investigated using flow cytometry. In Figure 5.1. it is seen that after one hour incubation, the efficiency of uptake increases with increasing number of tryptophans, until the addition of four tryptophans at the N-terminus, as seen by W_4R_8 showing lower uptake than W_3R_8. The uptake of RWR is higher than that of W_4R_8 and W_3R_8, but the highest uptake level is observed for RWmix. When incubated at 10 μM concentration, the fluorescence intensity of RWmix is almost ten times higher than that of W_4R_8. These results suggest that the number of tryptophans influence the uptake efficiency, and additionally, that the patterning of the tryptophan residues can have major effect on peptide transport across cellular membranes.

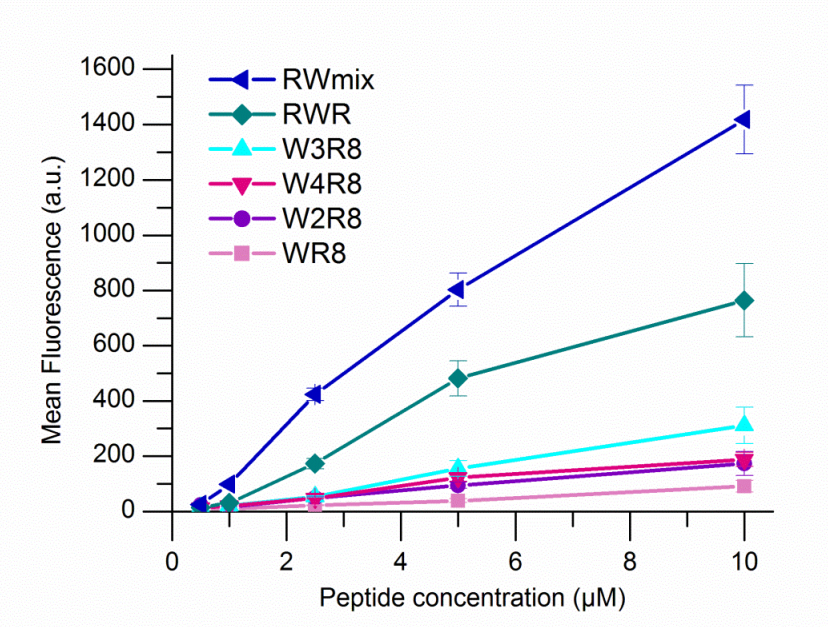

Figure 5.1. Cellular uptake of 5-FAM-labeled peptides into live CHO-K1 cells after 1 h incubation at 37 °C. The level of uptake increases with increasing amount of tryptophan, except for W_4R_8 which has an uptake comparable with W_2R_8. The two other peptides with four tryptophans (RWR and RWmix) show evidently higher uptake, with highest uptake observed for the peptide with evenly distributed tryptophans in the amino acid sequence, RWmix. Error bars are standard error of the mean and *n* is 6.

In addition to the quantity of internalized peptide, also the localization of the internalized peptide inside the cell is of interest when investigating cell-penetrating peptides since peptides that are localized in the nucleus can be used for transport of gene regulatory sequences. The intracellular localization of the six RW-peptides in live CHO-K1 cells was examined using confocal laser scanning microscopy imaging. Figure 5.2. shows uptake of the six RW-peptides after one hour incubation at either 37 °C or 4 °C. As can be seen in A, the staining pattern varies substantially between the different peptides, with punctate staining indicating, at least in part, endocytotic uptake for WR_8, W_3R_8, W_4R_8 and RWmix at 37 °C. W_4R_8 is mainly localized in vesicles close to the cell membrane. It is possible that this peptide somehow disturbs the endocytotic process and vesicles unable to "bud off" from the membrane are seen. W_2R_8 and RWR display weak punctate staining and strong nearly homogenous staining throughout the cytoplasm, with somewhat higher intensity in the cell nucleus. At 4 °C the punctate staining is absent (Figure 5.2., B). This is especially true for W_4R_8 which shows substantially decreased uptake with peptide mainly localized at the cell membrane. For the other peptides nearly homogenous fluorescence of the cytoplasm, and to some extent in the nucleus, is observed. This indicates that uptake takes place via both endocytosis and direct membrane transduction. The intracellular localization monitored for the RW-peptides seems to be dependent on the tryptophan position in the peptide sequence. These results point to W_2R_8 and RWR being the most efficient peptides for transport into the cell nucleus.

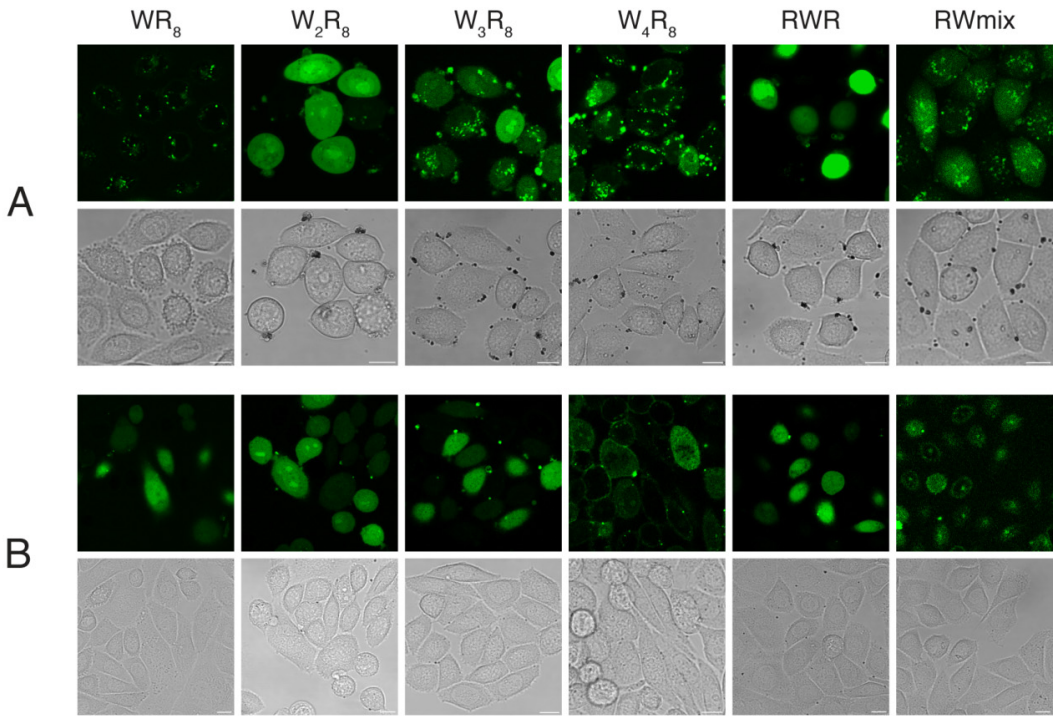

Figure 5.2. Confocal imaging of live cells incubated with fluorescein-labeled peptide for 1 h at either 37° C (A) or 4 °C (B). At 37 °C the fluorescence intensity vary between the peptides, with both punctate staining indicating endocytosis and, especially for W_2R_8 and RWR, diffuse uptake in the cytoplasm indicating direct penetration. At 4 °C the punctate staining is absent. N.B. Fluorescence intensities are not comparable due to optimization of gain for each picture.

5.2. The RW-peptides show low mammalian cell toxicity

A prerequisite for a well functioning CPP is a high therapeutic index, with high efficiency and low toxicity. CHO-K1 cells were incubated with peptide, at either 5 µM or 10 µM, and for one, two or three hours. Just before analysis, 7-AAD, a marker for dead cells, was added. As seen in Figure 5.3., the RW-peptides generally show low toxicity to mammalian cells. WR_8 and W_2R_8 are not more toxic than cell-medium only (negative control) and W_3R_8, RWR and RWmix show only low levels of toxicity. The exception is W_4R_8, which shows evident toxicity, especially at the higher peptide concentration after the long incubations. In addition, the peptides only caused minor leakage of POPC/POPG liposomes, meaning that they are not evidently membrane damaging. These results taken together with the high uptake of the RW-peptides, suggest that these peptides might be good candidates for therapeutic use. Especially RWR, which shows high uptake and low toxicity, and is found both in the cytoplasm and in the nucleus, might be a good candidate. Also W_2R_8, which shows a similar uptake pattern as RWR and simultaneously is not even slightly toxic, could be of interest.

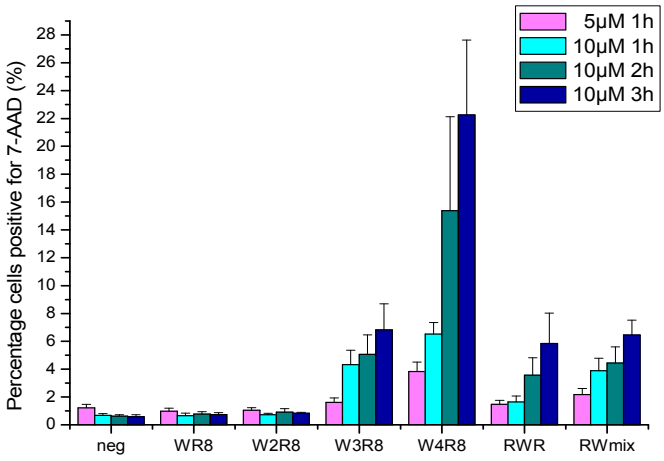

Figure 5.3. Toxicity of the RW-peptides to CHO-cells after 1, 2 or 3 hours of incubation at 5 µM or 10 µM. Only W_4R_8 is evidently toxic. Error bars are standard deviation of triplicates from three separate experiments.

5.3. The RW-peptides inhibit bacterial growth

Looking at the amino acid sequences of the RW-peptides, a resemblance to those of antimicrobial peptides can be seen. It was therefore tested if these cell-penetrating peptides could also inhibit bacterial growth. MIC, the minimal inhibitory concentration required for total growth inhibition in liquid medium, was measured on four bacterial strains, two gram positive (*S. aureus* and *S. pyogenes*) and two gram negative (*P. aeruginosa* and *P. mirabilis*) (Table 5.2.). The RW-peptides inhibit bacterial growth of the two gram positive strains at levels similar (*S. aureus*) and somewhat superior (*S. pyogenes*) to the well know lytic peptide melittin. For *S. aureus*, both W_4R_8 and RWR show a minimal inhibitory concentration of around 4 µM, and W_3R_8, W_2R_8 and RWmix show MICs of between 5 µM and 6 µM. In the case of *S. pyogenes*, W_4R_8 is evidently superior to the other peptides at inhibiting the bacterial growth, with MIC values almost ten times lower than for melittin. For both gram positive strains, the WR_8 peptide is evidently less potent than the other five peptides. The RW-peptides do not inhibit bacterial growth of the two gram negative bacteria *P. aeruginosa* and *P. mirabilis* within the concentration regime tested. Gram negative bacteria are generally more difficult to inhibit because of their more complex membrane structure, which might explain the result. The RW-peptides are, with the exceptions of WR_8, still efficient at inhibiting the growth of the two Gram positive strains tested, without causing evident membrane damage. These results point to the possibility that the RW-peptides may perform their anti-bacterial effect by binding to intracellular targets. The antibacterial effect seems to increase with increasing tryptophan content at the N-terminus, but since the effect of the peptides differs between the two Gram positive species tested, no clear connection regarding the position of the tryptophans can be seen.

Table 5.2. MIC (minimum inhibitory concentration) in μM for the RW-peptides and melittin.

	S. aureus	*S. pyogenes*	*P. aeruginosa*	*P. mirabilis*
WR_8	21	2.7	>43	>43
W_2R_8	5.4	0.59	>38	>38
W_3R_8	5.4	0.47	>34	>34
W_4R_8	4.4	0.12	>31	>31
RWR	3.9	0.79	>31	>31
RWmix	5.8	0.49	>31	>31
Melittin	5.6	1.4	22	>22

5.4. Secondary structures of the RW-peptides

It is plausible to think that the differences seen in cellular uptake, antimicrobial effect and mammalian cell toxicity are due to differences in the peptide secondary structure. To investigate whether the RW-peptides develop different secondary structure when interacting with lipid membranes circular dichroism measurements were performed. The result is depicted in Figure 5.4. and indicates that all the peptides except RWmix adopts a random-coil like structure, or extended structure, both when in solution and when interacting with POPC/POPG liposomes. In contrast, RWmix, also being random-coil in solution, adopts a helical-like structure when bound to liposomes, with maximum below 200 nm and minima at around 205 nm and 222 nm. The minimum seen at around 228 nm seen, especially evident for RWR and W_2R_8, is probably due to interactions between adjacent tryptophan residues.

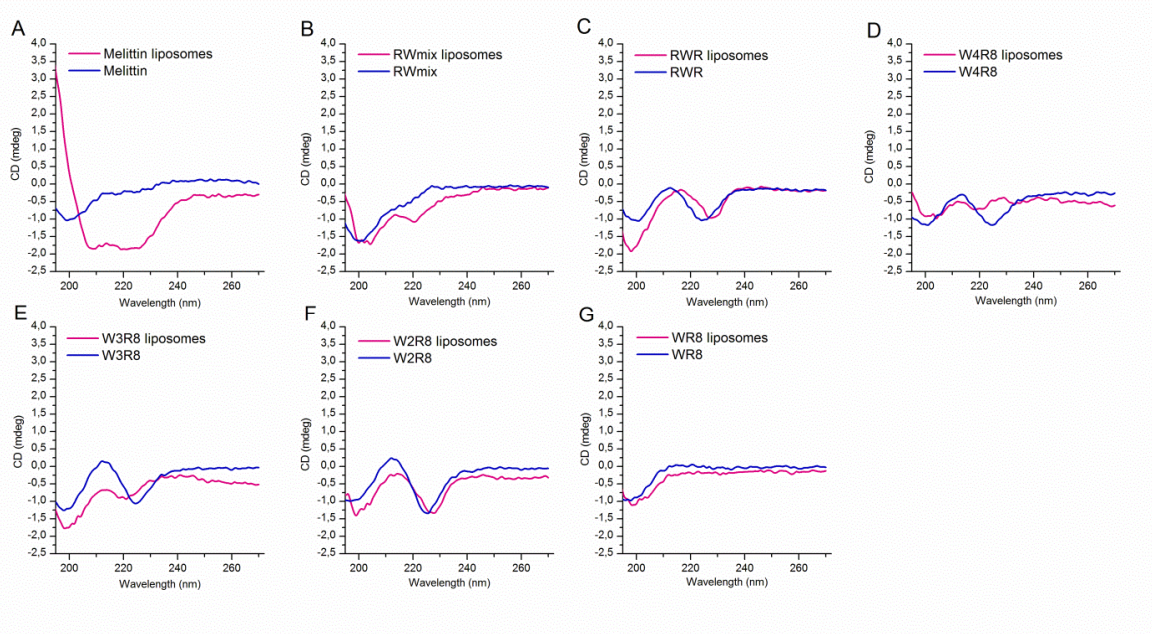

Figure 5.4. CD spectra of the six RWpeptides (B-G) free in solution (blue) and when bound to 80/20 mol% POPC/POPG liposomes (pink). As a comparison melittin (A), a well know lytic peptide that forms α-helix when interacting with lipid membranes is shown in A. RWmix forms a helical-like structure in combination with liposomes, the other RW-peptides are random-coils. The peptide to lipid ratio was 1:100.

To further examine the peptide secondary structure and to evaluate the orientation of the peptides relative the lipid membrane, linear dichroism was measured. Figure 5.5. shows the LD (left) and LD^r (right) for peptides combined with 80/20 mol% POPC/POPG liposomes. For simplicity only the three RW-peptides containing four tryptophans are shown. The LD and LD^r signals for W_4R_8 are very low, whereas RWR shows intermediate signals. For RWmix the LD and LD^r signals are instead relatively high, and this peptide thus has a higher orientation than the other peptides, both regarding allowed electric dipole transitions of the α-helix structure (around 209 nm for the long axis polarized transition) and the transition moments of tryptophan (around 225 nm, 270 nm and 290 nm). The other peptides, WR_8, W_2R_8, and W_3R_8 show LD signals of the same spectral shape and with amplitudes between those of RWR and W_4R_8. The higher orientation of RWmix, both of the tryptophans and the long helical axis, points to the possibility that this peptide has a helical-like structure, possibly with all the four tryptophans being oriented in the same direction. Furthermore, the LD^r absorption peaks at 209 nm (α-helix long axis) are, even if very weak, slightly positive, meaning that the peptides are situated parallel to the membrane.

The degree of molecular order and the secondary structure of the peptides can be coupled to their cell-penetrating properties. The peptide with the most pronounced order and secondary structure, RWmix, also has the highest uptake in CHO-cells. However, no obvious connection between the peptide secondary structure and the antibacterial effect could be seen.

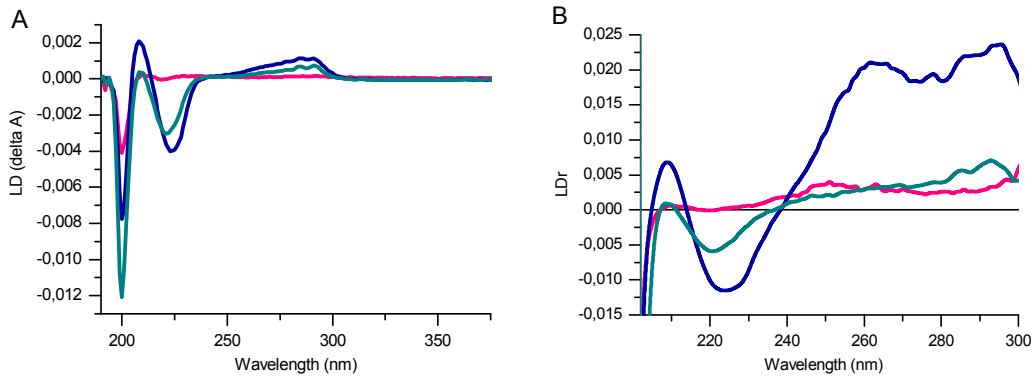

Figure 5.5. LD (A) and LD^r (B) spectra for W_4R_8 (pink), RWR (dark turquoise) RWmix (blue) when associated to liposomes consisting of 80/20 mol% POPC/POPG. The peptide-lipid-ratio was 1:100.

5.5. Probing the interaction between the RW-peptides and lipid membranes of different composition using QCM-D

The differences seen in the secondary structure and biological function between the RW-peptides, point to the possibility of different peptide-membrane interaction patterns. The differences were particularly pronounced for the three RW-peptides with the same number of tryptophans (four) but with varied tryptophan position in the peptide sequence. The interactions of these peptides with lipid membranes of different compositions were therefore

investigated using the quartz crystal microbalance with dissipation monitoring (QCM-D) technique. To the membranes, 5 μM of peptide was added during 20 min at a speed of 50 μl/min, whereafter the flow was stopped for 60 min. QCM-D has the advantage that it can quantify peptide binding to a membrane and monitor changes of the membrane that the peptide binding induces, without the need of labeling of either the peptide or the lipid components. In QCM-D, the peptide binding to the membrane surface is detected as a decrease in the frequency signal (Δf), whereas changes in the dissipation signal (ΔD) would indicate changes in the membrane.

In Figure 5.6. it is seen that the binding of RW-peptides to POPC membrane, as detected by QCM-D, is very low. The frequency shift is around or less than 0.5 Hz and also the dissipation signal is low. This illustrates the need of electrostatic interaction for efficient binding of the charged peptides to lipid membranes. The peptide binding to the POPC/Lactosyl PE membrane is somewhat higher than for the POPC membrane but still very low. This suggests that the peptides do not bind efficiently to short uncharged carbohydrate chains and that the lactose moiety sterically hinders electrostatic interactions between the negatively charged phosphate group of the glycolipids and the positively charged peptides. This further illustrates the need of electrostatic interaction for efficient binding to membranes.

When using negatively charged POPG/POPC membranes, the three peptides clearly bind. There seem to be small variations between the peptides both in the amount of peptide bound to the membrane and in the induced changes to the membrane, seen as differences in the maximum frequency signals (where W_4R_8 shows the largest mass deposition) and differences in the dissipation curves between the peptides respectively. When adding cholesterol to the membrane, these differences become more pronounced, with RWmix showing very different frequency and dissipations signals compared to those of RWR and W_4R_8. These differences possibly occur as a result of the α-helical structure of the RWmix peptide.

When the flow of peptide over the QCM-D sensor is stopped (second arrow), there is an increase in the frequency signal, in particularly for the POPC/POPG membrane. This might indicate a decrease in the mass deposition of the peptides, to an extent that differs between the peptides. This is a very unusual and unexpected result. As the peptide-membrane interaction seemingly had reached equilibrium, one would expect the signals to stay constant when the flow is stopped. If at equilibrium, one possible explanation to this finding could be a flow dependency in the interaction between the peptide and the membrane, leading to different shear forces. When the shear forces decrease as the flow is stopped, structural rearrangements may occur at the membrane surface. If the peptide–lipid membrane interaction is not at equilibrium, the varying decrease in mass deposition seen for the three peptides might be related to the peptides' different ability to cause reorganization and compactions of the membrane. RWmix would, due to its more pronounced secondary structure, cause more rearrangement of the membrane. This would lead to increased loss of water or lipid material, following a mechanism which was masked under the more efficient mass transport conditions during flow.

For the membrane with added cholesterol, the stop in peptide flow seems to have less effect and the interactions between the peptide and the membrane thus appear to be more stable, at least for RWR and RWmix. This might be explained by the lower mobility of this membrane and/or by specific interactions between the tryptophans and the cholesterols. Thus, the RW-peptides interact differently with membranes of different compositions and the secondary structure seems to be of particular importance in the interaction with cholesterol-containing membranes. The evident difference between RWmix and the other two peptides in the presence of cholesterol might be related to the uptake in mammalian cells, where both uptake efficiency and the degree of endocytotic uptake was higher for RWmix.

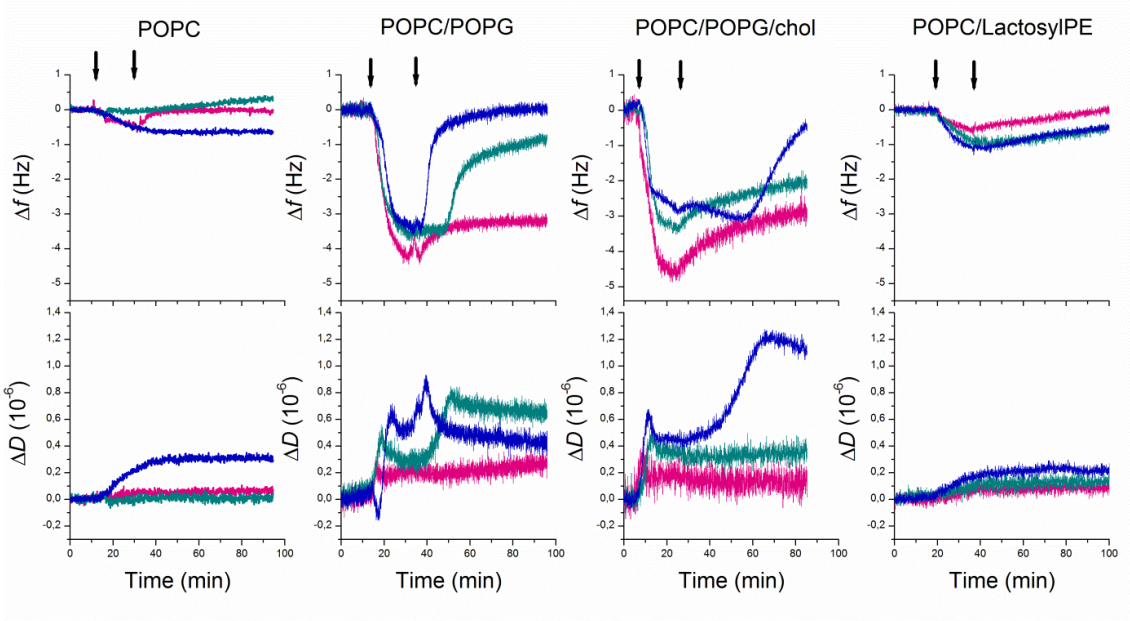

Figure 5.6. QCM-D monitoring of interactions between the three RW-peptides and bilayers of four different compositions: POPC, POPC/POPG 80/20 mole%, POPC/POPG/cholesterol 75/20/5 mol% and POPC/Lactosyl PE 80/20 mole%. RWmix is depicted in blue, RWR in turquoise and W_4R_8 in pink. For the POPC and POPC/Lactosyl PE bilayers peptide binding is low. For the POPC/POPG and POPC/POPG/cholesterol bilayers differences between the three peptides can be seen: RWmix in particular shows frequency and dissipation pattern evidently different from the two other peptides in the presence of cholesterol. First arrow indicates peptide addition, and second arrow indicates a stop in the flow of the peptide solution.

The mass depositions of RW-peptide on the lipid surface were verified by fluorescence quenching measurements, where fluorescently labelled peptide was titrated with increasing amount of POPC/POPG liposomes. In the left plot in Figure 5.7. it is seen that the fluorescence intensity shows a minima for all three peptides at peptide to lipid ratios of around 1 to 30. This means that at this peptide to lipid ratio the self-quenching is the highest and the peptides are positioned the closets to each other. From the minima values, maximum binding of peptide to the surface was calculated. The results show perfect correlation to the mass deposition values from the QCM-D measurements, verifying the accuracy of the

method. After the minima in fluorescence intensity, varying degrees of liposome addition are needed for the curves of the three peptides to level off. This correlates with the three RW-peptides' different ability to aggregate liposomes in solution, as seen in the right plot in Figure 5.7. Here, W_4R_8 aggregates liposomes at very low peptide concentrations whereas much higher concentration of RWmix is needed to observe the same degree of aggregation. These results suggest that not only the charge ratio between the positively charged peptides and the negatively charged liposomes but also the distribution of tryptophans and arginines in the peptide sequence affect the ability of the peptides to induce liposome aggregation.

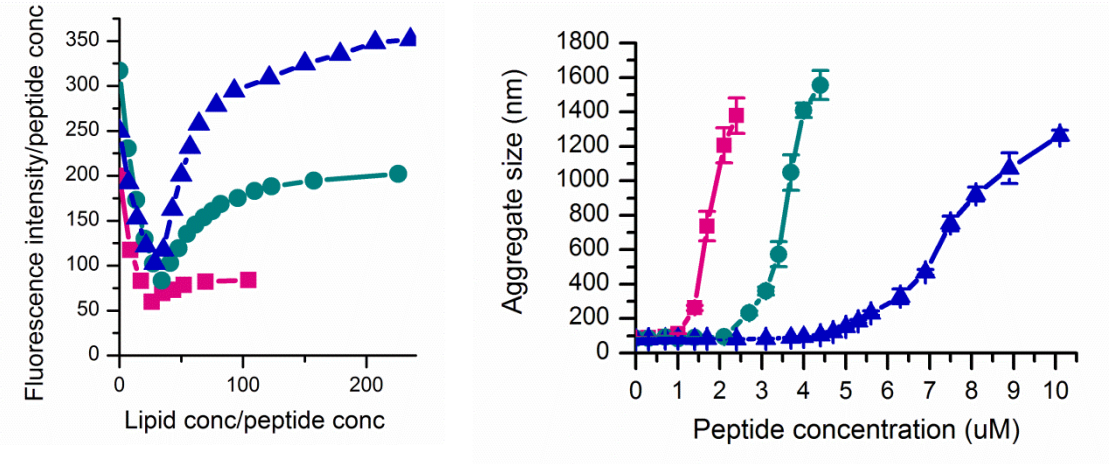

Figure 5.7. RW-peptide interaction with POPC/POPG 80/20 mole% liposomes. To the left fluorescence quenching of 5-FAM-labeled peptide as a function of increasing liposome concentration, showing that at a peptide to lipid ratio of around 1 to 30, the RW-peptides are the most closely packed since the fluorescence quenching is the highest. To the right approximate liposome aggregate sizes as function of increasing peptide concentration, showing that W_4R_8 is the superior of the RW-peptides at aggregating liposomes and that RWmix is the least aggregating peptide of the three.

6. Penetratin, two analogues and a homologue

Papers IV and V focus on penetratin-peptides and a penetratin-like peptide. In Paper IV, the aim was to investigate how arginine content and proteoglycans influence intracellular uptake and membrane binding of penetratin-peptides. Uptake and binding of the three peptides penetratin, PenArg and PenLys to wild type CHO-K1 cells and cells lacking proteoglycans were explored. PenArg and PenLys are analogues of penetratin, with either all the arginines or all the lysines substituted by each other (Table 6.1). In Paper V, the cell-penetrating and physical-chemical properties of a new CPP, the Oct4-PTD (Table 6.1), are described and compared with penetratin. Both papers also address the issue of how the fluorescent label may influence the peptides' properties. More thorough descriptions of methods and results are found in the amended papers.

Table 6.1. Amino acid sequences of penetratin, its two analogues PenArg and PenLys, and Oct4-PTD

Peptide	Sequence*
Penetratin	RQIKIWFQNRRMKWKK
PenArg	RQIRIWFQNRRMRWRR
PenLys	KQIKIWFQNKKMKWKK
Oct4-PTD	DVVRVWFCNRRQKGKR

*Arginines are shown in blue, lysines in turquoise and tryptophans in pink

6.1. Arginine content and cell surface proteoglycans affect uptake and binding of penetratin peptides

Membrane binding and cellular uptake were measured for penetratin and its two analogues PenArg and PenLys in wild-type cells (CHO-K1) and proteoglycan deficient cells (A745) using flow cytometry. Uptake was measured at 37 °C after one hour incubation, whereas membrane binding was measured after 15 min incubation at 4 °C, at which temperature endocytosis should be blocked. In Figure 6.1 it is seen that the presence of proteoglycans on the cell surface increases the peptide internalization and that both uptake and binding are favored by increasing arginine-content. Membrane binding (left column) and cellular uptake (right column) of PenLys are low and seem to be largely unaffected by the presence of proteoglycans. Penetratin in contrast, binds somewhat better to CHO-K1 than to the proteoglycan deficient cells, and the uptake of penetratin is evidently higher in the wild-type cells. PenArg, which shows both the highest binding and uptake, has clearly higher binding and uptake in the CHO-K1 cells at 5 μM, but not at 10 μM at which concentration the affinity and internalization appear to be similar in both cell lines. Thus, binding and uptake of PenArg seem to be favored by proteoglycans at the lower concentration, but not at the higher

concentration where binding and uptake levels are relatively equal. These results suggest that proteoglycans are not decisive for cell surface binding or uptake, yet binding affinity of arginine rich peptides is favored by the presence of proteoglycans on the cell surface. PenArg, which has the highest arginine content, also seems better at stimulating uptake when bound to the surface, which could be one explanation to its superior uptake.

The uptake experiments also revealed that fluorescently labeled PenArg is mildly toxic. At the higher concentration (10 μM), the peptide caused around 10 % dead cells in wild type cells and around 20 % in the proteoglycan deficient. Surprisingly, non-labeled PenArg is not toxic, illustrating how the fluorescent label may interfere with peptide properties.

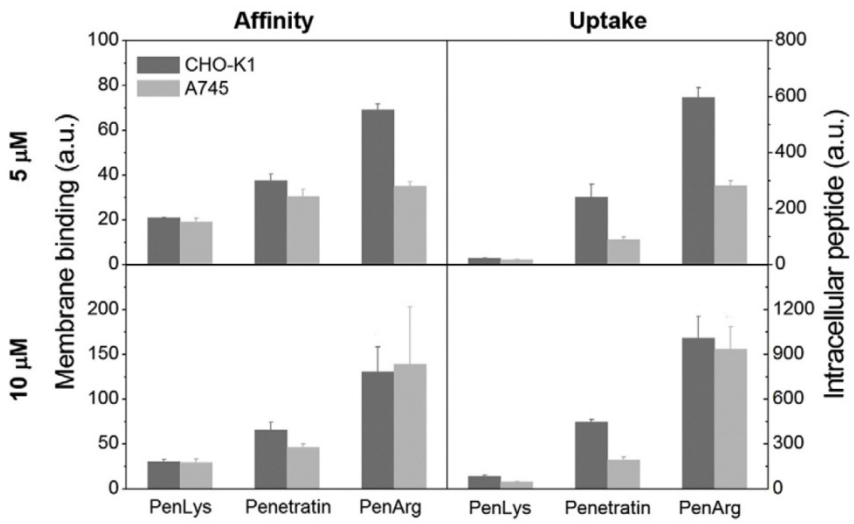

Figure 6.1. Cell membrane binding (left column) and uptake (right column) for 5 μM (top) or 10 μM (bottom) PenLys, penetratin and PenArg in CHO-K1 and proteoglycan deficient A745 cells. Both peptide binding and uptake into cells are favored by increased arginine content. Cellular uptake for the penetratin peptides is higher in the presence of proteoglycans, whereas peptide binding to the two cell types is similar. The exception is PenArg, which at 5 μM shows increased binding in the presence of proteoglycans and at 10 μM shows equal uptake in the two cell lines.

To further investigate how the arginine content may affect the peptides' interaction with cell surface proteoglycans, the ability of the three penetratin peptides to bind and cluster heparin was measured. Heparin, which is one of the glycosaminoglycan types of proteoglycans, is highly sulfated and has a very high negative charge density [210]. Fluorescently labeled peptide was titrated with increasing heparin concentration and fluorescence quenching was monitored. As seen in Figure 6.2, the degree of quenching is initially linear but levels off and reaches a plateau when charge neutrality between peptide and heparin is reached. At charge neutrality, the degree of quenching is the lowest for PenLys and the highest for PenArg. This suggests that the PenArg peptides are binding more tightly on the heparin molecules; a finding which also correlates with the formation of larger heparin-peptide aggregates. Thus,

the capacity to bind and aggregate heparin increases with increasing arginine content, possibly as a result of the bidentate binding of the guanidinium groups. This may thus explain the higher binding of PenArg to wild type cells.

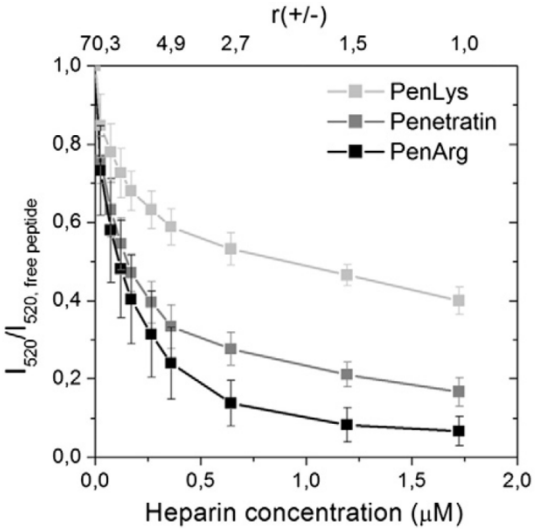

Figure 6.2. Binding of the penetratin peptides to heparin, shown as quenching of fluorescently labeled peptides as a function of increasing heparin concentration. The binding to heparin increases with increasing arginine content.

6.2. Oct4-PTD is a new CPP derived from a homeodomain protein

In Paper V, the new cell-penetrating peptide (also referred to as protein transduction domain, PTD), Oct4-PTD is explored. Oct4-PTD is the third helix of the Oct4 homeodomain protein, a transcription factor responsible for maintaining the pluripotent state of embryonic stem cells [211]. Because of the many similarities between Oct4-PTD and penetratin (e.g. cellular origin and amino acid sequence) it was investigated if Oct4-PTD might also be a cell-penetrating peptide. Cellular uptake, toxicity and secondary structure were compared between Oct4-PTD and penetratin. Oct4-PTD, which has a somewhat higher arginine content compared to penetratin, shows higher intracellular uptake, as monitored by flow cytometry. In addition, after one hour of incubation Oct4-PTD is localized in both the cell cytoplasm and nucleus, whereas penetratin is only found in the cytoplasm (Figure 6.3.). Localization in the nucleus is of major importance if a CPP is to be used as transporter of DNA-binding gene regulatory drugs, and the results suggest that Oct4-PTD might be a promising candidate for such use. The transport capability of Oct4-PTD was subsequently tested, showing that the peptide, in the form of the Oct4-PTD-Cre fusion protein, successfully translocates into cells where it causes gene activation.

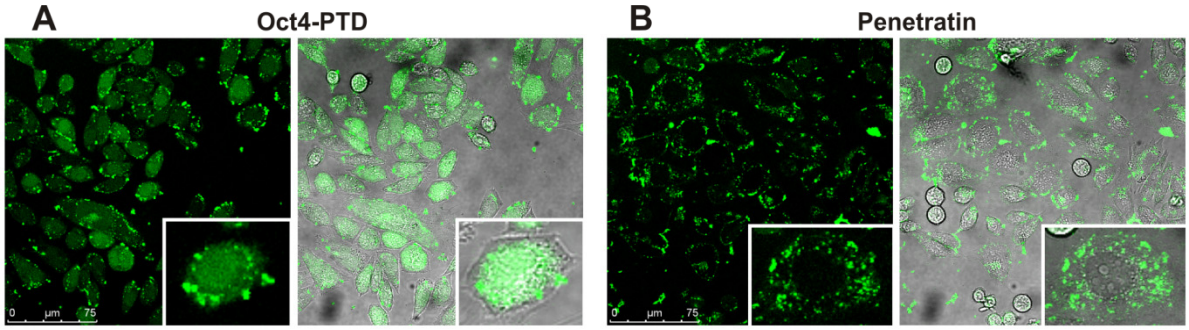

Figure 6.3. Cellular uptake of fluorescein isothiocyanate (FITC)-labeled Oct4-PTD (A) and penetratin (B) after one hour incubation.

Toxicity was measured to evaluate whether Oct4-PTD would be applicable as a CPP both *in vitro* and *in vivo*. Neither Oct4-PTD nor penetratin, which was used as comparison, showed any toxicity when unlabeled. However, the FITC-labeled peptides caused low levels of hemolysis, with somewhat higher levels for penetratin: 7.2 % compared to 2 % for Oct4-PTD at 45 µM concentration.

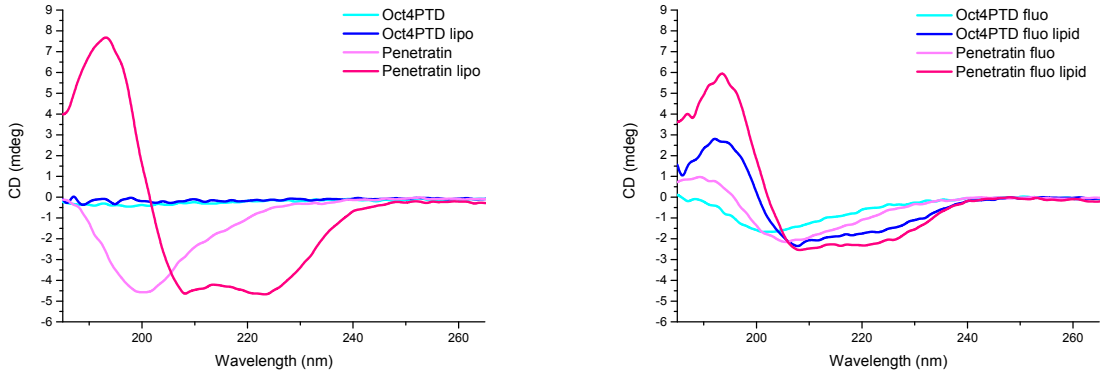

Figure 6.4. CD of Oct4-PTD (blue) and penetratin (pink), in solution (light colours) or associated with liposomes (dark colours). Without fluorescent label (left), penetratin changes structure from random-coil to an evident α-helix when binding to liposomes, whereas Oct4-PTD shows no secondary structure. With fluorescent label (right), both peptides adopt helical structures when associated with liposomes, and show random coils in solution.

Interestingly, the secondary structure of the peptide is also affected by the fluorescein label. In Figure 6.4. it is seen that unlabeled penetratin adopts random coil structure in solution and α-helical structure when associated with liposomes. Unlabeled Oct4-PTD seemingly shows no secondary structure at all. However, both the FITC-labeled peptides both adopt random-coils in solution and α-helixes when bound to liposomes. Thus, the fluorescein label might, depending on the peptide amino acid sequence, affect both peptide toxicity and secondary structure. This may be important to consider when working with CPPs.

7. Concluding remarks and outlook

Membrane-active peptides are promising candidates for the intracellular transport of drug molecules needed in gene therapy and for the treatment of the escalating occurring multiresistant bacteria. In this thesis it is shown that their efficiency is influenced by both the content of the amino acids arginine and tryptophan in the peptide sequence, and on cell membrane constituents like cholesterol and proteoglycans.

The cell-penetrating properties of a series of six arginine-tryptophan peptides gained from increasing tryptophan content. Fascinatingly, the patterning of tryptophans seemed to be of even greater importance. The cellular uptake was improved when the tryptophans were placed in the middle of the peptide sequence, as compared to at the N-terminus. The best uptake result was seen when the tryptophans were evenly distributed in the peptide sequence. Also the inhibition of bacterial growth was affected by the tryptophan position, but in contrast to the observation made for the cell-penetrating properties, the antibacterial properties gained from placing the tryptophans next to each other. The cellular uptake could be related to the peptides' secondary structure, with higher uptake correlating to a more pronounced secondary structure. No such connection could be seen regarding the antibacterial properties. The superior cellular uptake seen for the peptide with even distribution of tryptophans, might also be correlated to the higher degree of disturbance to cholesterol-containing membranes this peptide induces.

For the peptides in the penetratin-series, both intracellular uptake and membrane affinity increased with a higher content of arginines in the peptide sequence, both to wild type cells and cells lacking proteoglycans. In addition, the arginine-rich peptides were superior at binding to and clustering the proteoglycan analogue heparin. It was also seen that absence of proteoglycans at the cell surface reduced the uptake efficiency for both arginine-rich and lysine-rich peptides. These results accordingly suggest that proteoglycans are not decisive for cell surface binding or uptake, but that the presence of proteoglycans on the cell surface specifically favors the binding affinity and uptake of arginine rich peptides.

In this thesis a new cell-penetrating peptide, the Oct4-PTD, is also presented. It was shown to have higher uptake efficiency, but a less distinct helical structure, when compared to penetratin. The secondary structure and the toxicity were shown to be influenced by the fluorescent label. The impact of the fluorescent label also seemed to vary between the two peptides, suggesting an amino acid sequence dependency in this aspect.

As a next step, it would be interesting to investigate the membrane interactions of the tryptophan-arginine peptides further by including PE into the model membranes. By the addition of lipids with negative curvature, which have important membrane functions e.g. in endocytosis, new insights into how the tryptophan position affects cell-penetrating and antibacterial properties may be gained. It may for example be worthwhile to see if the

addition of PE can affect the induced changes to the membrane caused by peptide binding or even the capability to form pores.

Additionally, if the tryptophan-arginine peptides are to be used in the medical industry, it would be useful to further study their ability to transport cargo into cells. To date, these peptides have only been used for transport of the fluorescent label 5-carboxyfluorescein. For example, could their ability to transport enzymes into the cell cytosol or DNA into the cell nucleus be tested. Another approach could be to use the peptides for transport of lipid vesicles filled with drug molecules. Lipid vesicles are often used in cosmetics and drug delivery because of their biocompatibility. By use of membrane-active peptides, cellular uptake as well as cell specificity of lipid vesicles can be promoted.

In summary, membrane-active peptides are a group of peptides that shows great promise for multiple medical applications, such as gene therapy and treatment of multiresistant bacteria. In this thesis, the effect alterations in the amino acid content and membrane constituents may have on the activity of membrane-active peptides is studied. The results give insights to the mechanisms of action of arginine and tryptophan rich membrane-active peptides, and provide information for design of peptides with specified actions targeted for specific cellular membranes and intracellular locations.

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