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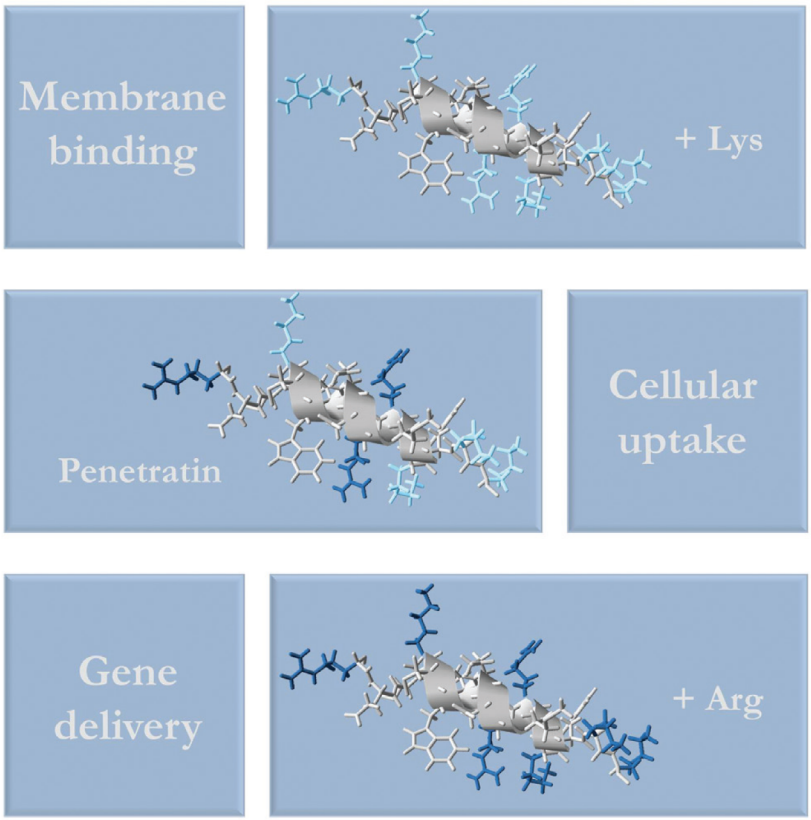

Cell-Penetrating Penetratin Peptides

Mechanistic Studies on Uptake Pathways and DNA Delivery Efficiency

HELENE ÅMAND

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2012

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Cover: Schematic illustration of the work performed in this Thesis.

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ABSTRACT

Delivery of gene-targeted drugs is limited by the inherently poor capacity of nucleic acids to overcome the membrane barrier of the cell, and development of vectors that can promote uptake is therefore crucial. *Cell-penetrating peptides (CPPs)* have emerged as promising vector candidates due to their ability to deliver a wide range of macromolecular cargos into cells. Uptake pathways of CPPs have been extensively studied for more than two decades, but we still lack a detailed mechanistic understanding of how CPPs interact with both the cell surface and the cargo to mediate delivery.

The work presented in this Thesis has increased our mechanistic insight into how CPPs function by addressing three key steps in CPP-mediated delivery: *cell surface binding*, *stimulated uptake* and *gene delivery efficiency*, using the classical CPP *penetratin* as a model peptide. The main focus is on the relative importance of the two cationic residues *arginine* and *lysine*, in order to rationalize the superior uptake often observed with arginine-rich CPPs. In addition, an important part of this Thesis is the development of a cell-like model system, *plasma membrane vesicles (PMVs),* for investigating CPP-cell surface interactions.

By studying both cell surface binding and internalization quantitatively, it is demonstrated that arginines have a greater capacity than lysines to bind to the cell surface and trigger internalization *via* macropinocytosis. Uptake efficiency is found to be dependent on each peptide's cell surface affinity, rather than on specific uptake-promoting interactions. However, arginines are also found to be less reliant on cell surface proteoglycans for internalization, and thus more versatile than lysines in promoting uptake *via* multiple pathways.

In addition to promoting uptake, arginines are also demonstrated to be superior to lysines in condensing DNA and mediating gene delivery. However, arginines alone are not sufficient, and hydrophobic residues are found to be necessary to stabilize CPP-DNA interactions. To improve gene delivery further, a strategy based on cysteine modification to allow formation of reversible CPP dimers is assessed. It is demonstrated that functionalization with a single cysteine residue has the capacity to improve stability, enhance endosomal escape and reduce cytotoxicity of CPP-DNA complexes. Altogether, the work presented in this Thesis provides important implications for design of peptide-based gene delivery vectors.

KEYWORDS: Cell-penetrating peptide, Penetratin, Uptake, Stimulated endocytosis, Cell surface affinity, Plasma membrane vesicle, Gene delivery, Arginine, Lysine, Cysteine

LIST OF PUBLICATIONS

This Thesis is based on the work presented in the following papers:

Paper I **Stimulated Endocytosis in Penetratin Uptake: Effect of Arginine and Lysine**
Helene L. Åmand, Kristina Fant, Bengt Nordén, and Elin K. Esbjörner
Biochemical and Biophysical Research Communications, **2008**, 371, 621-625

Paper II **Binding of Cell-penetrating Penetratin Peptides to Plasma Membrane Vesicles Correlates Directly with Cellular Uptake**
Helene L. Åmand, Carolina L. Boström, Per Lincoln, Bengt Nordén, and Elin K. Esbjörner
Biochimica et Biophysica Acta Biomembranes, **2011**, 1808, 1860-1867

Paper III **Cell Surface Binding and Uptake of Arginine- and Lysine-rich Penetratin Peptides in Absence and Presence of Proteoglycans**
Helene L. Åmand, Hanna A. Rydberg, Louise H. Fornander, Per Lincoln, Bengt Nordén, and Elin K. Esbjörner
Submitted to Biochimica et Biophysica Acta Biomembranes

Paper IV **DNA Condensation by Cell-penetrating Peptides and Relation to Their Ability to Mediate Transfection in Cell Culture**
Kristina Fant, Helene L. Åmand, Sofie Ståhl, Per Lincoln, Bengt Nordén, and Elin K. Esbjörner
Under revision for publication in Molecular Pharmaceutics

Paper V **Functionalization with C-terminal Cysteine Enhances Transfection Efficiency of Cell-penetrating Peptides through Dimer Formation**
Helene L. Åmand, Bengt Nordén, and Kristina Fant
Accepted for publication in Biochemical and Biophysical Research Communications, **2012**

CONTRIBUTION REPORT

Paper I	I performed and analysed the major part of the experimental work, and contributed to interpreting the results and writing the paper.
Paper II	I planned and performed most of the experimental work, analysed and interpreted all data and wrote the paper.
Paper III	I planned and performed the major part of the experimental work, analysed and interpreted all data and wrote the paper.
Paper IV	I took part in planning the study and performed the experimental work and data analysis together with the first author. I contributed to interpreting the results and writing the paper.
Paper V	I performed the experimental work, analysed and interpreted data, and wrote the paper together with the last author.

Publications not included in this Thesis:

Effects of Chirality on the Intracellular Localization of Binuclear Ruthenium(II) Polypyridyl Complexes
Frida R. Svensson, Johanna Andersson, Helene L. Åmand, and Per Lincoln
Accepted for publication in Journal of Biological Inorganic Chemistry, **2012**

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
A745	Chinese hamster ovary cell line mutant CHO-pgsA745
a.a.	Amino acid
ATP	Adenosine triphosphate
CD	Circular dichroism
CF	Carboxyfluorescein
CHO-K1	Chinese hamster ovary cell line
CPP	Cell-penetrating peptide
ct-DNA	Calf thymus DNA
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
FA	Formaldehyde
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
HEK293T	Human embryonic kidney cell line
HS	Heparan sulfate
LD	Linear dichroism
pDNA	Plasmid DNA
Peptiplex	Peptide-DNA complex
PG	Proteoglycan
PMV	Plasma membrane vesicle
RNA	Ribonucleic acid
TR	Texas red
siRNA	Small interfering RNA

TABLE OF CONTENTS

1. INTRODUCTION

A *cell* is the smallest functional unit of all living organisms. It simultaneously performs thousands of chemical reactions, which are ultimately regulated by the genetic code contained in the organism's DNA. Despite that the discovery of cells dates back more than three centuries [1], we are still far from a complete understanding of these remarkably complex entities. However, due to the rapid growth of molecular biology and genetics since the mid-20th century, our knowledge of cellular function has been greatly expanded down to the genetic level. This is also the key to understanding cellular *mal*functions resulting in disease and, importantly, opens up the possibility to use our genome as a drug target.

The human genome is estimated to contain approximately 25,000 protein-coding genes [2] and to date, mutations resulting in disease have been found in 2,700 of these genes [3]. Treatment for genetic disorders can in principle target either genetic or protein information, and both strategies involve transfer of large and complex biomolecules into cells; nucleic acids in the case of *genetic medicines* [4], and proteins in the case of *protein therapeutics* [5]. With these *biopharmaceuticals*, medicine is approaching a new era with tremendous opportunities to treat diseases in a highly specific manner that reduces the risk of adverse side effects. However, before actual clinical treatment the pharmaceutical industry must meet the challenges of poor cellular uptake, rapid degradation, and lack of stability commonly associated with biomacromolecules [6].

The membrane barrier of the cell constitutes a major obstacle for gene- and protein-based therapies and development of *vectors* that can mediate cellular uptake is therefore crucial. This is an intense area of research and current strategies include the use of recombinant viruses or synthetic vectors based on lipids, cationic polymers, nanoparticles or peptides [7, 8]. For gene delivery, viral-based vectors have completely dominated the scene, and out of the 1,700 clinical trials that have been approved so far, almost 70% involve the use of viral vectors [9]. The major advantage with viral-based vectors is their superior transfection efficiency compared to non-viral counterparts [10], but unacceptable side-effects have also raised serious concerns regarding their safety [11-13]. This clearly highlights the need for development of delivery vectors that are both efficient and safe.

Cell-penetrating peptides (CPPs) are a heterogeneous class of peptides with the intriguing capacity to enter cells and deliver macromolecular cargos in a non-toxic manner, making them highly interesting as non-viral delivery vectors. Despite the fact that the first CPP sequences were discovered more than two decades ago [14-17], clinical trials of CPP-based constructs were not initiated until 15 years later. Although still in early clinical development, more than 20 phase I/II trials have been performed to date [18] and in

addition, numerous CPP-constructs for disease treatment have been described in both *in vivo* and *in vitro* models [19]. Despite this promising development, we still lack a detailed mechanistic understanding of how these peptides enter cells and deliver functional cargo. Today, endocytosis is recognized as a major pathway, especially when CPPs are coupled to large cargos, but direct penetration through the membrane is also suggested for some CPPs (see references [20-22] for recent reviews). A full comprehension of the molecular prerequisites that lead to internalization of CPPs through different routes is important for the future design of efficient peptide-based vectors.

The focus of this Thesis is fundamental aspects of CPP function and the work presented in the appended papers aims at understanding the physico-chemical basis of membrane interactions, uptake and gene delivery efficiency (as outlined in Figure 1.1). At centre of attention is the classical CPP penetratin and its two arginine- or lysine-substituted analogs PenArg and PenLys, designed to investigate the relative importance of these two basic amino acid residues. In *Papers I-III*, cellular uptake of penetratin peptides is investigated and explanations for the differences in uptake efficiency are sought in terms of capacity to bind to the cell surface and to evoke a biological uptake response. *Paper I* explores the ability of these peptides to internalize into mammalian cells and trigger their own uptake by stimulating endocytosis. In *Paper II,* the relative affinity for the cell surface is investigated using a membrane model system that is novel in CPP research, namely plasma membrane vesicles (PMVs), derived directly from the plasma membrane of mammalian cells. *Paper III* addresses the role of cell surface proteoglycans in CPP uptake, by investigating the influence of these sulfated sugars on both cell surface binding and uptake efficiency. In *Papers IV-V*, focus is shifted from CPP interactions with membranes to interactions with DNA and the penetratin peptides are assessed with respect to their potential for gene delivery. *Paper IV* combines biophysical characterization of peptide-DNA complexes ("peptiplexes") with transfection in live cells. Finally, *Paper V* explores the strategy of cysteine incorporation to improve peptiplex stability and endosomal escape. It is demonstrated throughout this Thesis that lysine-to-arginine substitution in penetratin results in a peptide with high capacity to bind to the cell surface, stimulate endocytic uptake, and mediate gene delivery, which are all key functions for an efficient cell-penetrating peptide.

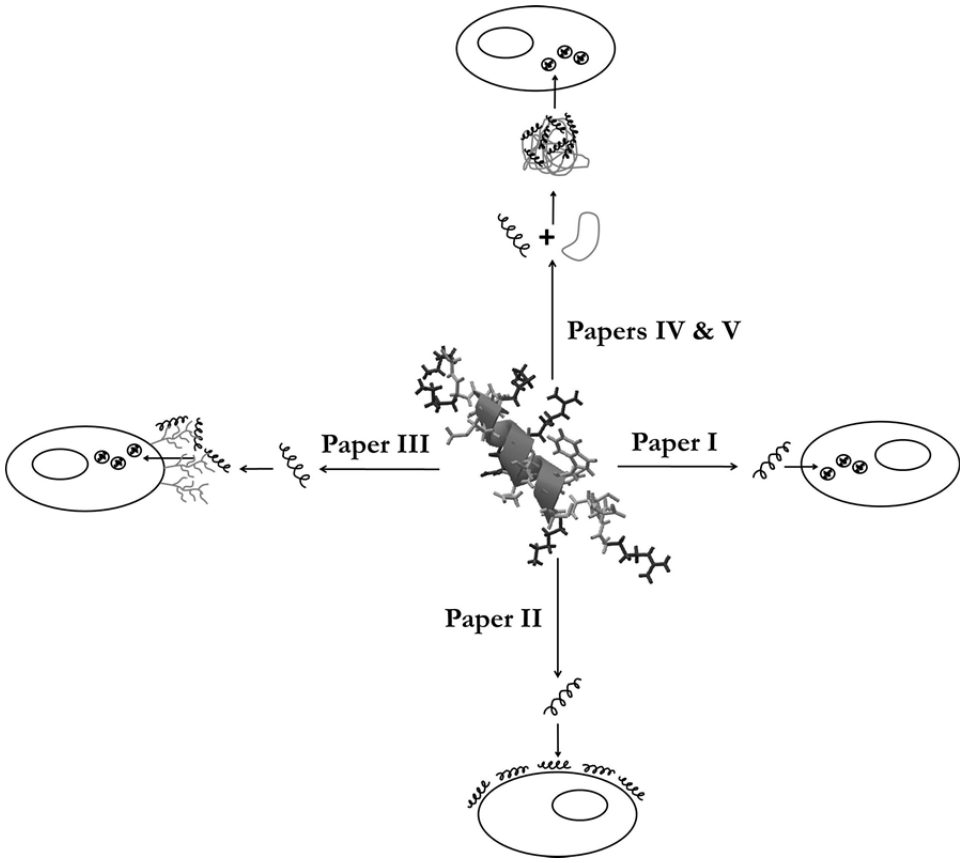


Figure 1.1 Overview of Papers I-V. *Paper I* explores uptake and endocytic stimulation, *Paper II* membrane binding affinity, *Paper III* the role of proteoglycans in cell surface binding and uptake, and *Papers IV-V* gene delivery efficiency of penetratin peptides.

2. BACKGROUND

This Chapter provides the background and context for the research described in this Thesis. The CPP field is described from a historical perspective, with focus on their uptake mechanisms and their use as DNA delivery vectors.

2.1 CELL-PENETRATING PEPTIDES

About two decades ago, our knowledge about cell membrane impermeability was challenged by the discovery that certain proteins and peptides appeared to enter cells *via* an energy-independent mechanism[1] [14-17]. These findings became the foundation of a new research field, involving the study of the uptake mechanism and applications of these so-called *cell-penetrating peptides (CPPs),* also sometimes referred to as *protein transduction domains (PTDs)*. Today, more than hundred CPP sequences are known but despite this, CPPs as a class is still not precisely defined due to difficulties in determining their translocating mechanism and large variations in both sequence and secondary structure. The general definition today is that "*CPPs are relatively short peptides, 5-40 amino acids, with an ability to gain access to the cell interior by means of different mechanisms, including endocytosis, and with the capacity to promote the intracellular delivery of covalently or non-covalently conjugated bioactive cargoes"* [23]. Due to the lack of detailed mechanistic understanding, membrane interactions and uptake mechanisms of CPPs have been areas of intense investigation ever since the discovery of these peptides. Today, these fundamental studies are performed in parallel with the development of CPP-based therapeutic strategies *in vivo* [24].

2.1.1. History and Overview

The fact that positively charged peptides can enter cells has been known since the 1960's [25]. However, the real starting point for research on cell-penetrating peptides was two independent discoveries in 1988, showing that the 86-residue transcription-activating protein *Tat*, involved in replication of the human immunodeficiency virus HIV-1, could rapidly translocate over cell membranes [14, 15]. A few years later this was demonstrated also for the 60-residue *Antennapedia* homeodomain protein from *Drosophila* [16] and in 1994, a 16-residue sequence from the third helix of this protein, denoted $Antp^{43-58}$ or *penetratin*, was demonstrated to be responsible for its cell-penetrating properties [17]. Research in this area has grown rapidly ever since these initial discoveries were made, and

[1] Since translocation was observed to occur also at 4°C and after ATP depletion.

more than hundred peptide sequences have been shown to enter cells and deliver macromolecular cargoes, including plasmid DNA, siRNA, proteins and nanoparticles, both *in vitro* and *in vivo* [26]. These peptides differ substantially, both with respect to their origin and their primary sequences, but have certain features in common, including a high content of positively charged and/or hydrophobic amino acid residues. CPPs have been divided into subgroups based on either origin (*protein-derived*, *chimeric* or *synthetic*) [26] or sequence characteristics (*primary-*, *secondary-* or *non-amphipathic*) [27]. Table 2.1 lists a number of common CPPs according to both classifications.

Table 2.1 Classification, sequences and origin of some common CPPs and their derivatives. Positively charged amino acids are marked in bold and hydrophobic are underlined.

Family	Name	Sequence†	Class#	Origin
Protein-derived	Penetratin (Antp$^{43-58}$)*	**R**Q**K**IWFQN**RR**M**K**W**KK**	sa	*Drosophila* homeodomain Antennapedia
	Tat$^{47-57}$*	YG**RKKRR**Q**RRR**	na	HIV-1 protein Tat
	pVEC	LLIIL**RRR**I**RK**QAHAHS**K**	sa	Vascular endothelial cadherin
Chimeric	Transportan*	GWTLNSAGYLLG**K**INL**K**ALAALA**KK**IL	pa	Galanin and mastoperan
	MPG (DeliverX^{TM})*	GALFLGFLGAAGSTMGAWSQP**KKKRK**V	pa	Fusion sequence of HIV gp41 and NLS of SV40 T-antigen
	Pep-1 (Chariot^{TM})	**K**ETWWETWWTEWSQP**KKKRK**V	pa	Reverse transcriptase of HIV and NLS of SV40 T-antigen
Synthetic	Octaarginine (R8)*	**RRRRRRRR**	na	Positively charged sequence
	MAP	**K**LA**L****K**LA**L****K**A**L****K**AA**L****K**LA	sa	Amphipathic model peptide
	TP10	AGYLLG**K**INL**K**ALAALA**KK**IL	pa	Deletion analog of transportan
	PenArg*	**R**Q**R**IWFQN**RR**M**R**W**RR**	sa	Penetratin analogs substituted with Arg/Lys
	PenLys*	**K**Q**K**IWFQN**KK**M**K**W**KK**	sa	
	EB1*	LI**R**LWSHLIHIWFQN**RR**L**K**W**KKK**	sa	Penetratin analog designed to be endosomolytic

* Peptides used in the work of this Thesis
† From N-terminal
# Classes of CPPs: pa; primary amphipathic, sa; secondary amphipathic, na: non-amphipathic

When classified in terms of origin, the largest family is the *protein-derived* CPPs, which include the already mentioned penetratin [17] and Tat [28] peptides. Another example from this family is pVEC, which is an 18-residue peptide derived from the vascular endothelial cadherin protein, involved in cell adhesion [29]. *Chimeric* peptides are formed by fusing peptide sequences from different origins. For example, several CPPs are based on a signal sequence that directs insertion of intracellular proteins into the membrane of specific organelles, combined with a nuclear localization signal (NLS). MPG is a 27-residue peptide formed from the fusion sequence of HIV-1 gp41 (involved in viral fusion with the host cell), coupled to the NLS sequence of the Simian virus 40 (SV40) T-antigen [30]. The same NLS sequence combined with the HIV-1 reverse transcriptase was used to construct the 21-residue CPP Pep-1 [31]. Both MPG and Pep-1 are today commercially available under

the trade names DeliverX™ and Chariot™ for *in vitro* delivery of siRNA and proteins, respectively. Another commonly used chimeric CPP is the 27-residue transportan, which was incidentally discovered by Langel *et al.* while searching for novel galanin receptors [32]. Transportan is a fusion peptide consisting of the neuropeptide galanin and the wasp venom toxin mastoparan [33]. *Synthetic* CPPs include both completely *de novo* designed sequences based on certain chemical traits believed to favour peptide internalization, and modifications of sequences with natural origin. Examples of synthetic CPPs are peptides designed to form amphipathic α-helices, such as the model amphipathic peptide MAP [34], or Tat-inspired oligoarginines [35, 36]. Other examples are a number of deletion analogs of transportan that have been developed to avoid the potential inhibitory effect that transportan exerts on membrane GTPases. Notably, transportan 10 (TP10) has been successful in many applications [37]. Other examples of designed CPPs include the penetratin analogs PenArg, PenLys and EB1. PenArg and PenLys are arginine- and lysine-substituted penetratin versions, designed to specifically study the relative importance of these basic residues [38] and are central to the work described in this Thesis. EB1 (endosomal breaker 1) was developed to avoid entrapment in endosomes following endocytic uptake, by formation of an α-helix that could penetrate the endosomal membrane upon endosome acidification [39]. CPP design is often performed on a trial-and-error-basis, but algorithms that can predict CPP sequences based on knowledge of existing CPPs have also been developed and has been reviewed by Hansen *et al.* [40].

As mentioned above, CPPs can also be classified according to their amphipathic character and binding properties to lipid membranes, as reviewed by Ziegler [27]. *Primary amphipathic* CPPs (paCPPs) includes *e.g.* transportan and MPG and typically contains more than 20 amino acids with an alternating pattern of hydrophobic and hydrophilic domains in their primary sequence. These peptides interact readily with both neutral and anionic lipid membranes. The *secondary amphipathic* CPPs (saCPPs), including penetratin peptides and pVEC, are generally shorter than paCPPs and reveal their amphipathic character first upon formation of α-helix or β-sheet structures that are induced by their binding to membranes. This class of peptides typically interact with membranes containing a certain fraction of anionic lipids. *Non-amphipathic* CPPs (naCPPs), such as Tat or R8, are generally short with a high or even exclusive content of cationic amino acids. This group of peptides does not interact with model lipid membranes unless they contain a relatively high proportion of anionic lipids. The sequence characteristics and membrane interactions of CPPs may to some extent be linked also to their uptake mechanism(s), which are further discussed in the following section.

2.1.2. Mechanisms of Cell Internalization

The mechanism(s) by which CPPs can enter cells has puzzled the CPP field for years and is, despite numerous studies, still not completely resolved. This is also a subject of some controversy, and contradictory results can be found in the literature. Our current understanding of CPP uptake mechanisms have been summarized in recent reviews by for example Madani *et al.* [20], Trabulo *et al.* [21], and Heitz *et al.* [22]. From a historical perspective, research on CPP uptake has shifted from originally being focused on finding

one common mechanism employed by all CPPs, towards a gradual apprehension that different mechanisms can be utilized depending on both CPP and cargo properties, and that several mechanisms are even likely to act in parallel. Given the heterogeneity in CPP sequence characteristics, it is perhaps not surprising that these peptides use different routes of entry into the cell.

The original definition of a CPP was a peptide that could traverse biological membranes by a passive diffusion mechanism, independent of temperature, ATP, and receptors. CPP uptake was then considered as a pure physico-chemical phenomenon and peptide translocation was therefore extensively investigated in lipid model systems with attempts to explain how a charged peptide could cross a hydrophobic lipid membrane [41-50]. However, in 2002, the CPP field took a drastic turn when it was discovered that earlier cell studies on CPPs could suffer from artefacts due to redistribution of plasma membrane-associated peptide during cell fixation and insufficient removal of externally bound peptide in cells analysed by flow cytometry [51, 52]. This lead to a re-evaluation of CPP uptake mechanisms using live cell protocols and careful removal of externally bound peptide. Today, endocytosis is recognized as a major pathway, especially when CPPs are coupled to large cargos [53-56]. Hence, CPP internalization has become more of a biological phenomenon that cannot be described solely by peptide-lipid interactions. Although focus has shifted towards endocytic mechanisms and these will most likely be involved in therapeutic applications of CPPs, direct translocation is still considered as a possible uptake route for some CPPs. Indeed, direct membrane penetration of cationic peptide segments is an intriguing physico-chemical phenomenon on its own and has attained considerable attention also outside the CPP field. The most prominent example is perhaps the suggestion that membrane insertion of arginine-rich domains in the KvAP K^+ channel is decisively regulating its function [57, 58]. Both direct penetration and endocytosis can occur through a number of different mechanisms, which are depicted schematically in Figure 2.1 and will be described briefly below.

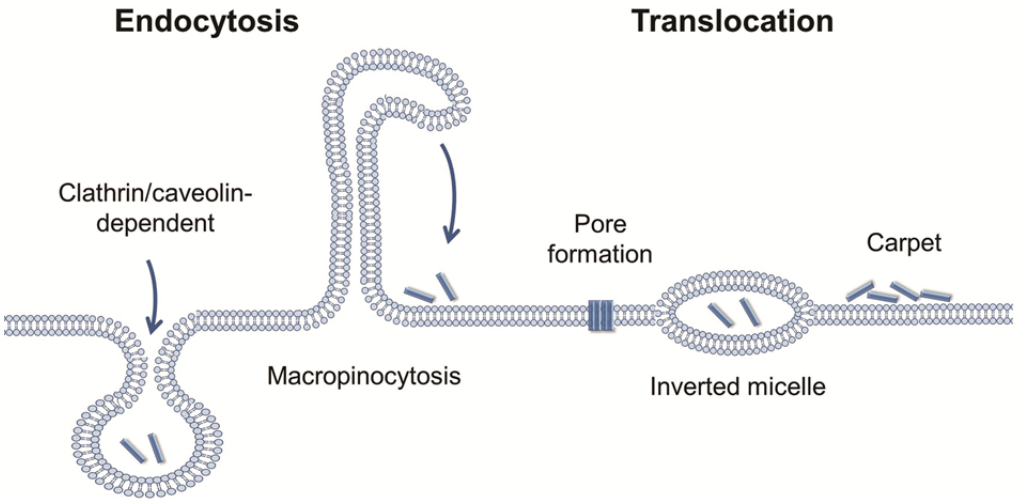


Figure 2.1 Putative uptake mechanisms for CPPs.

Direct translocation has been proposed to occur through a number of different mechanisms, described by the *inverted-micelle*, *pore formation* or *carpet models* [20, 21]. The *inverted-micelle model* was initially described for penetratin [59] and involves destabilization of the membrane upon peptide binding, leading to formation of inverted hexagonal structures (micelles), with peptide being located in the hydrophilic environment of the micelle core. In addition to electrostatic interactions between positively charged CPPs and negatively charged membrane components, hydrophobic interactions are also suggested to be important for this mechanism. *Pore formation* can be described either by the *barrel stave* or *toroidal models*, which were originally presented to describe membrane interactions of anti-microbial peptides (reviewed in [60]). Both models involve formation of transient pores as peptides are inserted into the membrane and oligomerized into ring-shaped structures. In the *barrel stave model* pores are formed by amphipathic α-helical peptides, with the hydrophobic face interacting with the lipid chains and the hydrophilic face forming the interior of the pore. In the *toroidal model* the pores are similar, but the lipids are bent so that the peptide only interacts with the polar headgroups, thereby resulting in significant rearrangement of the lipid bilayer. The *carpet model* has also mainly been suggested for anti-microbial peptides [60] and involves accumulation of peptide at the bilayer surface and destabilization of the membrane in a detergent-like manner, eventually leading to membrane disruption.

As discussed in the previous section, the amphipathic nature of a CPP has been proposed to influence its interactions with, and hence capacity to translocate across, the membrane [27]. Thermodynamic studies have shown that primary amphipathic CPPs are able to bind and insert into lipid membranes already at low peptide concentrations. This is suggested to enhance uptake *via* direct translocation mechanisms, but also to result in higher toxicity. By contrast, membrane insertion of secondary amphipathic CPPs requires a change in secondary structure, whereas binding of non-amphipathic CPPs occurs mainly to the headgroup region and membrane translocation and perturbation is therefore expected to take place first at elevated peptide concentrations [27]. In live cells, direct translocation has been observed mainly for CPPs having a high content of either hydrophobic residues [61-63] or arginines [38, 55, 64-67]. Whereas membrane translocation of hydrophobic peptides is conceivable, penetration of highly charged arginine-rich peptides seems less intuitive. Therefore, in order to account for direct translocation of cationic CPPs, a model based on charge neutralization by interaction with hydrophobic counterions such as anionic lipids has been proposed [68-72]. In support of this model, it has been shown that cellular uptake of arginine-rich CPPs can be enhanced in presence of hydrophobic counterions such as pyrenebutyrate [73, 74].

Endocytosis can mechanistically be viewed upon as a two-step process where molecules are first internalized into lipid vesicles (termed *endosomes*), followed by escape from these endosomes and entry into the cytosol. Endocytosis consists of several mechanistically diverse pathways, including *macropinocytosis, clathrin-mediated, caveolin-mediated and clathrin/caveolin-independent endocytosis*, which are more thoroughly described in section 3.3.2. Despite the large body of literature clearly demonstrating the involvement of endocytosis in CPP uptake, the contribution from each pathway remains elusive and one predominant

mechanism is unlikely to be established. Instead, different routes of entry, both endocytic and non-endocytic, appears to be utilized depending on the nature of both the CPP (sequence, conformation, length) and the cargo (type, size, binding methodology), but also on experimental conditions such as cell type and peptide concentration with respect to cell density [20, 22].

To understand CPP uptake from a mechanistic perspective, much effort has been put into identifying cell surface binding sites that could act as mediators of CPP internalization. Due to their positive charge, CPPs interact electrostatically with a variety of cell surface anions, including different proteins, lipids and sugars. However, few of these molecules have been systematically investigated in the context of CPP uptake, and most focus has been on cell surface *proteoglycans (PGs)*, especially those containing *heparan sulfates (HSPGs*). These glycoproteins are heavily substituted with sulfated carbohydrates, and thus possess a high negative charge density (described in section 3.3.1.). In addition to their polyanionic nature that facilitates binding through electrostatic attraction, HSPGs are known to be involved in endocytosis of protein ligands [75, 76]. It has also been suggested that cationic CPPs may induce clustering of HSPG chains at the cell surface, resulting in initiation of intracellular signalling cascades that trigger endocytosis [76, 77]. However, such clustering events have only been observed *in vitro* in bulk experiments with soluble sulfated sugars [78-81]. Comparative studies of CPP uptake in wild-type and PG-deficient cells are frequently encountered in the literature. CPP uptake is, in general, significantly reduced but not completely abolished in the absence of PGs [55, 82-89]. Most of these studies have focused on oligoarginines or else arginine-rich peptides such as Tat, but PGs have also been shown to promote uptake of the hydrophobic transportan analog TP10 [89], and are suggested to be involved in uptake of MPG [90]. However, despite seemingly compelling evidence that PGs are involved in internalization of CPPs, their exact role in mediating uptake is still elusive. Clearly, the negative charge of these sulfated sugars provides unspecific electrostatic attraction for cationic CPPs, but in addition, a more specific receptor-like role has been suggested, as certain CPPs bind selectively to proteoglycans with particular patterns of sulfation [76, 77, 84, 91-94]. On the other hand, recent studies have pointed towards that proteoglycans may in fact have a less prominent role in uptake of oligoarginines [95] and cargo transduction of Tat [86], and for Tat the role of membrane proteins was instead emphasized.

Twenty years of research has certainly expanded our knowledge of CPP uptake, but has also made its complexity increasingly clear. Today, we still lack definite answers to several key questions, including how different physico-chemical properties of CPPs determine their corresponding uptake pathway(s) (*i.e.* clear structure-activity relationships), what interactions on the cell surface that elicit an uptake response, and how CPPs escape across endosomes and deliver functional cargo. The fact that these important pieces of the puzzle are still missing limits the successful development of CPPs as intracellular delivery vectors.

2.1.3. Penetratin Peptides

The classical CPP penetratin and its two arginine- or lysine-substituted analogs PenArg and PenLys, have been used throughout the work presented in this Thesis. Earlier studies with these peptides are therefore summarized below. Recent literature reviews of membrane interactions, uptake mechanisms and cargo delivery of penetratin can also be found in work by Dupont *et al.* [96, 97] and Alves *et al.* [98].

As previously mentioned, penetratin was one of the first CPPs to be discovered. This 16-residue peptide is derived from the *Drosophila Antennapedia* protein, which belongs to a group of transcription factors called homeoproteins. These proteins regulate transcription by binding to DNA *via* a highly conserved motif called the homeodomain. DNA binding involves three α-helices, with the third helix being responsible for recognition and binding to the DNA major groove [99]. It was also this helix that was found to mediate translocation of the Antennapedia protein [17]. Penetratin was originally reported to enter cells by an energy-independent pathway, with tryptophans suggested to be critical for the translocation process [17]. Further studies concluded that uptake was independent of both chiral membrane receptors and α-helical structure, since D-analogs, inversed or scrambled sequences, and penetratin versions substituted with proline residues to break α-helicity, were all efficiently internalized [59]. A direct translocation mechanism based on formation of inverted micelles was proposed [59] and found some support from nuclear magnetic resonance (NMR) studies showing that interaction of penetratin with membranes can result in formation of inverted structures [100]. Several structure-activity relationship (SAR) studies have clearly demonstrated that basic residues are critical for uptake of penetratin [38, 101-103]. The importance of hydrophobic residues, in particular the two tryptophans, is less clear, and SAR studies both support [103] and reject [38] their involvement in penetratin uptake.

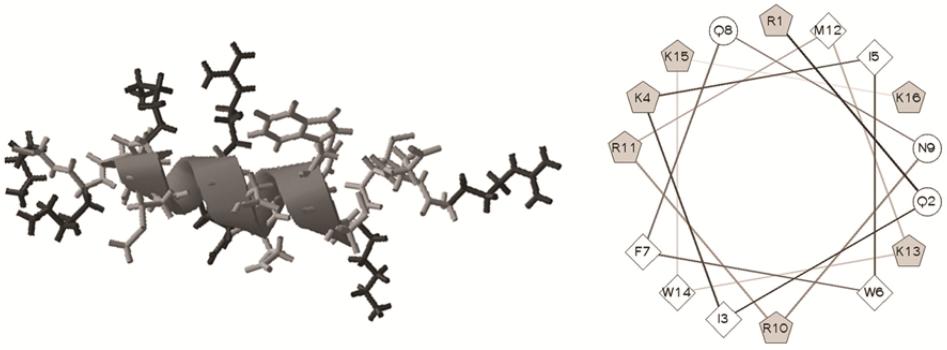


Figure 2.2 Structure of penetratin when bound to lipid membranes. The molecular coordinates are adapted from the 1OMQ structure in the Protein Data Bank, deposited by Lindberg *et al.* [44]. Helical wheel representation with basic residues depicted in grey is shown to the right.

In order to obtain a physico-chemical explanation for its membrane translocative properties, penetratin has been extensively studied in lipid model systems. Penetratin interacts readily with anionic lipid membranes and binds with a parallel orientation to the membrane surface with only limited bilayer insertion [43-45, 102]. In addition, membrane

binding leads to a structure transformation from random coil to an amphipathic α-helix (Figure 2.2) [43, 44, 102-106], but penetratin also appears to possess conformational plasticity which allows it to transform to β-sheets at higher peptide/lipid ratios [104, 105]. Interestingly, this secondary structure was recently reported to be predominant in cells [107]. Despite these observations, ordered secondary structure content does not seem to promote uptake. By contrast, even a negative correlation between α-helical content and internalization efficiency has been proposed [43]. Translocation of penetratin across lipid bilayers has also been investigated in different lipid model systems. The first direct observations of penetratin translocation was reported in 2000, using fluorescence microscopy to explore internalization into giant unilamellar vesicles (GUVs) [50]. Since then, several conflicting results have been reported [41, 46, 49], but translocation has also been observed in the presence of a transmembrane potential [42, 48]. The ability of penetratin to translocate across a pure lipid bilayer thus appears to be dependent on model system.

As described in the previous section, CPP research has today shifted focus from direct translocation mechanisms towards endocytosis, following the discoveries of potential artefacts in earlier work [51, 52]. For penetratin, the initial reports on direct translocation has also been re-evaluated but since live cell studies have both confirmed [38, 108] and rejected [88, 109] involvement of direct translocation the matter remains unresolved. Membrane translocation has been suggested to exclusively occur within limited concentration regimes, but also here the reports are incoherent, with translocation observed either at low [88, 110] or high penetratin concentrations [55]. However, although direct translocation may still contribute to penetratin uptake, a vast number of live cell studies today point towards different endocytic pathways as major uptake routes for penetratin [38, 55, 108]. In addition, involvement of proteoglycans has been suggested in several studies [83, 88, 89, 110] and penetratin have also been shown to cluster such sulfated sugars *in vitro* [79, 81]. In summary, it thus seems like penetratin can use different routes of entry into cells, even though we still lack a complete mechanistic understanding of this process. Nevertheless, penetratin has been shown to enter cells even when coupled to macromolecular cargos and has been successfully used for delivery of *e.g.* peptides, proteins, oligonucleotides and siRNA (see ref. [97] for a comprehensive review). Today, penetratin is also commercially available with an N-terminal pyridyl disulfide function, allowing conjugation *via* disulfide bridges to cargos carrying thiol groups (*e.g.* cysteine residues) [111].

In 2003, our group expanded the penetratin family with two new members [38]; the arginine- or lysine-substituted analogs PenArg and PenLys (Figure 2.3). It had previously been shown that arginines were fundamentally important for the function of Tat and furthermore that oligoarginines could enter cells more efficiently than oligolysines [35, 64]. With PenArg and PenLys, the arginine versus lysine comparison was extended also to penetratin, which compared to Tat normally has a higher lysine-to-arginine ratio and, in addition to positive charge, also contains hydrophobic residues. A live cell study involving this penetratin triplet of peptides indicated that arginine substitution promotes endocytic

uptake into cells and also provides a certain ability to enter cells through non-endocytic routes [38]. Lysine substitution initially appeared to completely abolish uptake [38], but the extensive characterization described in this Thesis shows that PenLys is actually weakly internalized (section 5.1.). The penetratin peptides have also been thoroughly investigated in lipid model systems in order to explain the large dissimilarities in cellular uptake from a physico-chemical perspective. Arginine/lysine-substitution creates a penetratin peptide with similar structure, orientation and insertion depth into lipid bilayers [43], but arginines do enhance binding affinity for anionic membranes [43, 47], and counterion-mediated partitioning into a hydrophobic phase [68]. A higher capacity to cause local lipid rearrangement has also been suggested [43]. However, these differences are not large enough to account for the accentuated differences in peptide internalization into cells, and the focus of this Thesis is therefore to search for explanations for the superior uptake capacity of arginines on a molecular level.

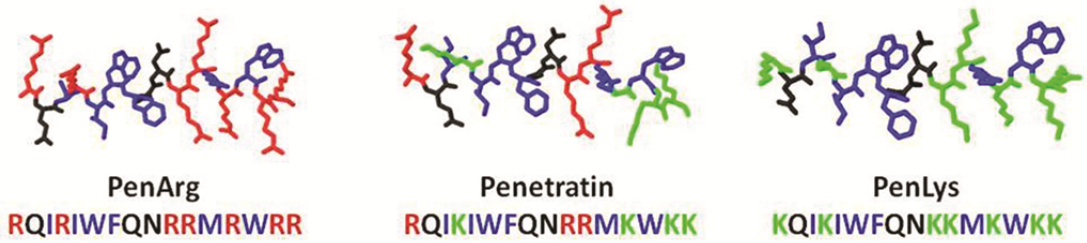


Figure 2.3 Amino acid sequences of penetratin peptides. Arginines are marked in red, lysines in green and hydrophobic residues in blue.

2.1.4. CPP-Mediated Delivery of Nucleic Acids

Nucleic acids are promising drug candidates due to their ability to specifically target disease at the genetic level. However, successful pharmacological application of these molecules is highly dependent on development of non-toxic delivery vectors that can provide efficient protection from degradation and promote both internalization into target cells and subsequent release in the cytoplasm or nucleus. Nucleic acid-based strategies include both short sequences such as small interfering RNAs (siRNAs) and antisense oligonucleotides (ONs) that interfere with gene expression by targeting mRNA in the cytoplasm, and plasmid DNA containing thousands of basepairs for delivery into the cell nucleus and expression of therapeutic genes [21, 22].

CPPs have been successfully used to deliver nucleic acid cargos [112, 113]. Most of these applications have involved delivery of short oligonucleotides, and relatively few have reported delivery of entire plasmids. In early studies, the most common approach was to covalently attach cargo to the CPP through various linkers, but in recent years non-covalent strategies based on condensation of DNA through electrostatic interactions have gained attention due to the relative ease and higher versatility associated with this method. In addition, while covalent CPP technology provides high reproducibility and control of the stoichiometry of the CPP-cargo constructs, a major limitation with this approach appears to be the risk of altering the biological activity of the cargo [22]. Covalent

strategies have been used for neutral oligonucleotides such as peptide nucleic acids (PNAs) [114-116]. However, non-covalent complex formation with peptide in excess may be more promising for negatively charged cargos such as siRNA, since the negative charges otherwise mask the cationic peptide charges and thus perturb CPP function [117]. In addition, non-covalent attachment is essentially a requirement for delivery of plasmid DNA, due to the large size and high number of negative charges [112].

The first CPP to be extensively explored for non-covalent delivery was the chimeric peptide MPG. It has been used in this way to deliver both oligonucleotides [30, 61] and plasmid DNA [118]. Non-covalent approaches have been extended also to other CPPs, but the main limitations for efficient delivery appear to be lack of peptiplex stability and entrapment of cargo in endocytic vesicles. Different strategies to avoid these limitations have therefore been investigated in many studies. For Tat, the transfection efficiency has for example been shown to be enhanced by formation of multimers or branched Tat variants [119-121], most likely due to an improved capacity to condense DNA. In addition, incorporation of hydrophobic groups such as stearyl chains increase delivery of both plasmids and oligonucleotides using oligoarginines [122-124], and oligonucleotide delivery with the transportan analog TP10 [125]. This approach has been suggested to stabilize peptiplexes and improve membrane interactions to facilitate both uptake and endosomal escape [122, 123]. Another strategy involves incorporation of an endosome-disruptive sequence, which has been shown to enhance penetratin-mediated delivery of siRNA [39]. An alternative interesting approach to CPP-mediated gene delivery involves combination of CPPs with other gene delivery methods such as liposomes, polyethyleneimine or nanoparticles, in order to combine efficient packaging, delivery and targeting [21]. One of the most sophisticated systems is the so-called Multifunctional Envelope-type Nano Devices (MEND), which are virus-inspired assemblies consisting of a polycation-condensed DNA core, coated with a lipid envelope functionalized with octaarginines [126, 127].

In summary, a vast number of reports have clearly demonstrated the potential of cell-penetrating peptides as delivery vectors for therapeutic biomolecules such as nucleic acids. Today, several pre-clinical and clinical trials are already underway [18, 22], even though we still lack detailed mechanistic insight to several fundamental questions regarding CPP function. The work presented in this Thesis aims at answering some of these questions, by exploring the effect of amino acid substitution on membrane interactions, uptake pathways and cargo delivery capacity of CPPs. Combining such mechanistic studies with continued efforts to reach clinical trials will hopefully make way for therapeutic use of cell-penetrating peptides.

3. FUNDAMENTAL CONCEPTS

This Chapter describes the fundamentals of biological macromolecules and cellular membranes, important for the work in this Thesis

3.1. PEPTIDES

Peptides are the central molecules in this Thesis, and this section aims at describing the structure and function of these biopolymers from a general perspective. Peptides and proteins (*i.e.* polypeptides) have the dual role of being both main *building blocks* and *working machinery* of living cells, executing nearly all cellular functions. This functional heterogeneity implies a chemical and structural diversity and this is provided by the sequence of amino acid building blocks.

The general structure of an amino acid, with a so called α carbon being attached to an amino group, a carboxyl group and a side chain (R), is shown in Figure 3.1A. In the physiological pH range, both the carboxyl and the amino group will be charged and the amino acid is thus a zwitterion. Amino acids in peptides and proteins are covalently linked together through formation of an amide bond between the carboxyl and amino groups of two adjacent amino acids. The unreacted amino and carboxyl groups at each end are called N- and C-terminus respectively. Many proteins have N-termini blocked with an acetyl group and a few also have C-termini modified to amides [128]. Such modifications are also common in synthesis of peptides, to avoid contributions from terminal charges. A peptide sequence is commonly reported by its one- or three-letter amino acid abbreviations, written with the N-terminus to the left and C-terminus to the right.

The amide bond, or more commonly *peptide bond*, has substantial double bond character and as a consequence, the O, C, N and H atoms are essentially coplanar, *i.e.* lie in the same geometric plane. Polypeptides usually fold into regular *secondary structures*, out of which the most common are the right-handed *α-helix* and the parallel or anti-parallel *β-sheet*. Peptide regions with less defined structures are collectively denoted as *random coils*. The secondary structures of proteins are commonly studied by X-ray crystallography or nuclear magnetic resonance (NMR), but can also be measured using spectroscopic techniques. Circular dichroism (see section 4.1.3.) is for example a standard technique for determination of secondary structure content of soluble proteins and also for monitoring conformational changes such as folding/unfolding reactions.

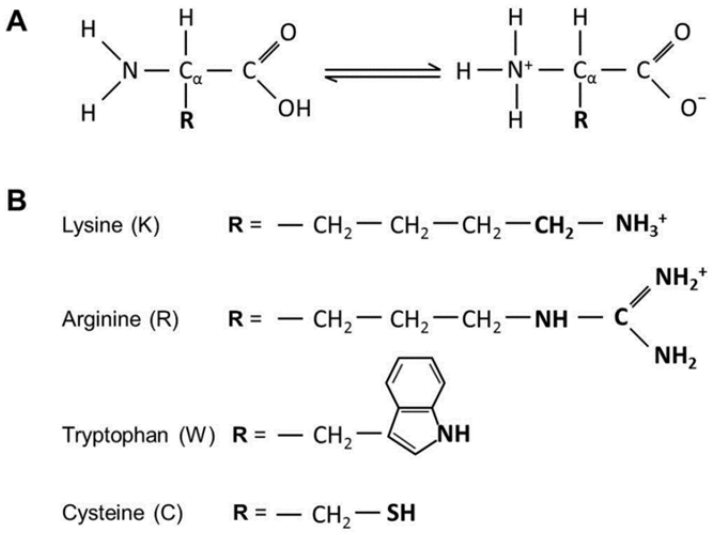


Figure 3.1 Structure of α amino acids. (A) General backbone structure and formation of zwitterion. **(B)** Structure of side chains relevant for the work of this Thesis. Note that the positive charge of arginine is delocalized over the three nitrogens.

As mentioned above, the properties and structure of peptides and proteins are depicted by their amino acid sequence. The amino acid alphabet contains 20 different letters, all with different side chain composition and thus different chemical properties. Based on the nature of the side chain, amino acids are divided into acidic, basic, polar or hydrophobic. A few amino acids are of particular importance for the work in this Thesis and that is first of all the basic amino acids *arginine (R)* and *lysine (K)*, which are two of the most common residues found in CPP sequences and have been shown to be decisive for their function [35, 101, 103, 129]. These both contain ionisable nitrogen-containing groups, amine in the case of lysine and guanidinium in the case of arginine (Figure 3.1B). With pK_a values above 9, these side chains are positively charged at physiological pH. Arginines and lysines thus carry identical charge and have only minor differences in chemical structure. Yet, the guanidinium group of arginine side chains have the ability to form particularly stable bidentate hydrogen bonds with oxo-anions such as phosphates or sulfates and consequently, arginines have been suggested to possess an enhanced capacity to interact with these biomolecules, compared to lysines [130]. The amino acid alphabet contains also a third basic residue, namely *histidine (H)*, but since it has a pK_a around 6, histidine is only positively charged in acidic environments.

A second class of amino acids that has attracted some particular attention in CPP research are the *aromatic* amino acids, phenylalanine (F), tyrosine (Y) and tryptophan (W). Due to their conjugated rings, these residues all have hydrophobic character and furthermore absorb and emit light in the UV region, making them possible to study by optical spectroscopic techniques (see section 4.1.). *Tryptohan* has become the primary chromophore in spectroscopic studies of proteins and peptides due to its environment-sensitive photophysical properties. Tryptophan emission is sensitive to solvent polarity and thus functions as an intrinsic reporter of environmental changes that can be used to study *e.g.* protein folding or peptide-membrane binding (see reference [131] for examples).

Tryptophan is also interesting since it has been suggested to be involved in mediating uptake of CPPs [17, 101, 103].

The last amino acid that plays a role in the work of this Thesis is *cysteine (C)*. The side chain of cysteine contains a *thiol* (SH) group that can form a *disulfide bridge* with another cysteine side chain through oxidation. These bonds play an important role in folding and stability of proteins and are further utilized in *Paper V* of this Thesis to form CPP dimers.

3.2. DNA

DNA is a *nucleic acid* and thus belongs to another major class of biomacromolecules. Nucleic acids perform the fundamental task of storing and transmitting *genetic information* and can thus be regarded as the most central molecules for life itself. Indeed, the discovery of the molecular structure of DNA was awarded the Nobel Prize in 1962 [132]. In this Thesis, the role of DNA is as a *cargo* molecule, *i.e.* DNA is used to investigate the capacity of CPPs to deliver macromolecules into cells. This section will present the basic structure and function of DNA and further describe condensation of DNA for gene delivery purposes.

There are two types of nucleic acids, *ribonucleic acid (RNA)* and *deoxyribonucleic acid (DNA)*. They both consist of a polymeric chain, built up from monomeric units called *nucleotides*, connected by covalent bonds. In DNA, the nucleotides in turn consist of a five-carbon *sugar* (deoxyribose), a negatively charged *phosphate* group and one of four possible nitrogen *bases*: adenine (A), thymine (T), cytosine (C), or guanine (G) (Figure 3.2A). While alternating sugars and phosphates are structural components forming the DNA *backbone*, the bases hold the *genetic code*, *i.e.* they contain the information needed to make and maintain a living organism. The key to this intriguing property is the ability to perform *self-duplication*. This is in turn due to the specific *base-pairing*, *i.e.* formation of hydrogen bonds, between A and T or C and G respectively. However, to describe the molecular basis for DNA function, the basic structure of the DNA molecule first needs to be considered.

Under physiological conditions, DNA exists as a double-stranded, right-handed helix with two identical, but anti-parallel strands (Figure 3.2B). While the hydrophilic and highly negatively charged sugar-phosphate backbone faces the aqueous surroundings, the bases are directed towards the centre of the helix, perpendicular to the helix axis. This configuration also gives rise to two spiral grooves, the wide major groove and the narrow minor groove. The helical structure is stabilized by hydrophobic interactions between the stacked bases and by hydrogen bonding between bases on opposite strands. Due to the hydrogen bonding pattern of the four bases, this base-pairing is highly specific, which implies that the two strands are complementary to one another. This is also the key to the self-replicating property of DNA that is used both to copy the genome during cell division (*i.e. replication*), and to produce complementary single-stranded RNA molecules that can subsequently be used as templates to direct protein production (*i.e. transcription* and *translation*). These fundamental processes are together known as the central dogma of molecular biology [133].

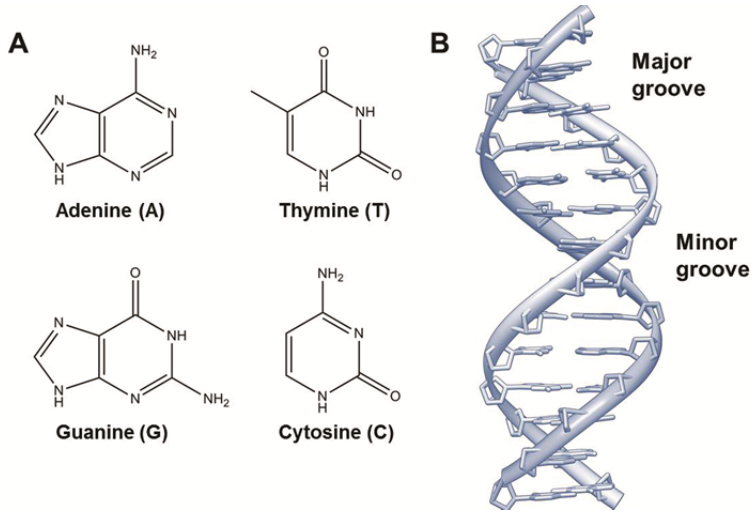


Figure 3.2 Structure of DNA. (A) The four DNA bases **(B)** The DNA double helix.

3.2.1. DNA Condensation for Gene Delivery

Condensation of DNA is of vital importance *in vivo*. The most striking example is packaging of the genome into chromosomes, where approximately 2 m of DNA is confined to a μm-sized cell nucleus. A similar decrease in DNA volume can be obtained also *in vitro* using chemical agents, *e.g.* for gene delivery purposes. DNA condensation *in vitro* is a large research field on its own and is not a major focus of this Thesis, but the basics of this process will be briefly described below as they are of importance for the work presented in *Papers IV* and *V*.

In aqueous solution, DNA exists as a random coil due to repulsion between the negative charges in the phosphate backbone. Hence, efficient transport of DNA over the hydrophobic plasma membrane (section 3.3.) requires reduction of both size and charge. During *condensation* of DNA, the random coil structure is transformed to a state where the volume fraction of DNA and solvent are comparable and the helices might be separated by only a few layers of water. Although condensation of single molecules has been observed, usually several DNA molecules are incorporated into the same particle. It can thus be difficult to clearly distinguish condensation from aggregation, but the term condensation is often defined as formation of aggregates with a finite size and ordered morphology [134, 135].

Many different substances can be used for condensation of DNA and in all cases, condensation is achieved either by decreasing the repulsion between the DNA segments or by making DNA-solvent interactions less favourable. Charge neutralization of the phosphate backbone can for example be achieved by cations, whereas solvent interactions can be perturbed by adding a poorer DNA solvent or a polymer that excludes volume and results in molecular crowding. Additionally, multivalent cations may also facilitate condensation by causing local distortion or bending of the helix structure [135]. Condensation of DNA is a result of a rather complex interplay between different interactions, including the electrostatic forces between DNA and its counterions or

different DNA segments, entropy loss upon collapse into an ordered state, entropy gain from released counterions, and bending stiffness of the helix that limits the maximum curvature [134].

3.3. THE MEMBRANE BARRIER

Biological membranes constitute a major obstacle for intracellular delivery vectors such as CPPs and interaction with and uptake over the plasma membrane is the main focus of this Thesis. This section presents the basic structure and function of biological membranes, describes mechanisms for transport over the membrane and finally discusses the use of membrane models to study membrane interactions.

3.3.1. Cellular Membranes

Cells are enclosed by a *plasma membrane* that serves as a protective *barrier* and maintains the essential differences between the cell interior (cytosol) and the extracellular milieu. In eukaryotic cells, membranes are also used for *compartmentalization* by separating membrane-enclosed organelles such as the nucleus, mitochondria, endoplasmatic reticulum (ER), and Golgi apparatus from the cytosolic environment. The exact membrane composition can vary significantly with cell and organelle function, but all membranes have a common structure that can be described as a thin film of *phospholipids* and *protein* molecules, held together mainly by non-covalent interactions. Lipids, out of which phospholipids are the most abundant type, form the matrix of membranes and typically constitute 50 mass percent of animal cell membranes [136]. The amphipathic nature of phospholipids, with polar head groups and hydrophobic hydrocarbon tails, results in that lipids are highly prone to self-associate into energetically favoured *bilayers*, where the hydrophobic tails are sandwiched between the hydrophilic heads and thus shielded from water (Figure 3.3). Apart from phospholipids, the membrane also consists of varying amounts of sterols, with cholesterol being the major sterol component in mammalian cells. Cholesterol has a structure consisting of four rigid rings, which partly immobilizes the hydrocarbon tails and therefore reduces membrane permeability. Membrane proteins are embedded into the lipid matrix either as peripheral or membrane-spanning and perform most of the specific functions of cell membranes, such as cell signalling and membrane transport.

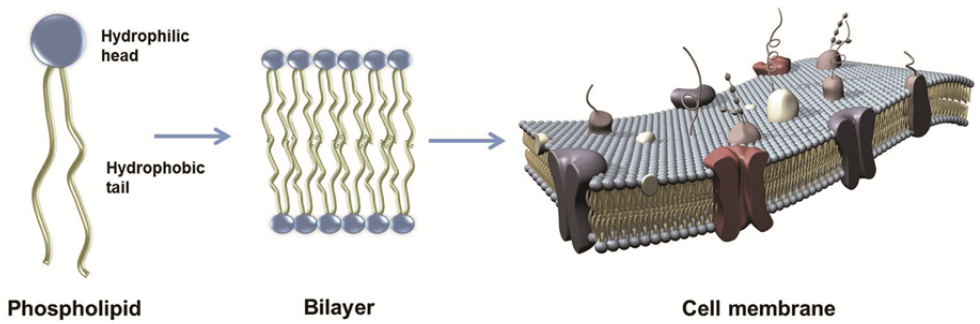


Figure 3.3 Schematic illustration of phospholipid structure, bilayer formation and membrane organization.

The cell membrane may contain hundreds of different lipid species, entailing its structural and functional complexity [137, 138]. Originally, the lipid portion of the membrane was described as a continuous fluid phase, in which membrane proteins are randomly dispersed (*the fluid mosaic model* from 1972 [139]). However, during the last decades it has become increasingly clear that the lateral motion of membrane components is more restricted, and that membranes may contain regions of variable thickness and with accumulation of certain lipids and proteins [140, 141]. These microdomains, or *lipid rafts*, enriched in sphingolipids, cholesterol and particular proteins have gained considerable interest for their possible function in cell signalling and regulation [142, 143], and have also been suggested to be involved in uptake of CPPs [144]. The lipid bilayer is not merely a passive barrier, but in addition to its protective role lipids take an active part in cell function, *e.g.* by enabling membranes to change shape and thereby perform budding, tubulation, fusion and fission and, furthermore, as first and second messengers in signal transduction [137, 138].

The plasma membrane is not only laterally segregated, the distribution of membrane components between the intra- and extracellular leaflets is also asymmetric. This asymmetry is functionally important, since many cytosolic and extracellular proteins or ligands bind specifically to certain lipid headgroups [145]. In eukaryotic plasma membranes, anionic lipids such as phosphatidylserine (PS) and phosphatidylinositol (PI), are mainly distributed in the inner leaflet, while *e.g.* sphingomyelin and sugar-containing glycolipids are exposed to the extracellular environment [138, 146]. Lipid asymmetry is not static, and loss of lipid asymmetry to expose PS in the outer leaflet can for example be triggered by external stimuli and is an early marker for apoptosis [147].

In addition to lipids and proteins, the cell surface is also rich in sugars, out of which many are negatively charged. These oligosaccharides are covalently attached to both lipids (*glycolipids*) and proteins (*glycoproteins*) and together form a carbohydrate coat on the membrane called the *glycocalyx*. A group of glycoproteins that has received particular interest in CPP research are the *proteoglycans (PGs)*, which have been suggested to have a receptor role in uptake [76, 77]. They are distinguished from other glycoproteins by the nature, arrangement and quantity of sugar chains. First of all, PGs must by definition contain at least one *glycosaminoglycan (GAG)* chain. GAGs are built up from chains of alternating amino sugars and uronic acids that are highly sulfated and also contain carboxyl groups. This makes GAGs the most anionic molecules produced by animal cells and due to this polyanionic nature they have, from a physico-chemical perspective, been described as an extracellular equivalent to DNA [76]. In contrast to the short, branched oligosaccharides found on most glycoproteins, GAGs are long (typically 80 residues) and unbranched. Proteoglycans are heavily glycosylated with up to 95 mass percent carbohydrates attached to a *core protein,* that is either inserted across the bilayer or coupled to a glycosylphosphatidylinositol (GPI) anchor. PGs as a group possess almost limitless diversity due to different core proteins, variations in number and type of attached GAG chains and also the pattern of sulfation of these chains.

Proteoglycans are not only diverse in terms of structure, but also in terms of function and their physiological importance is illustrated by the severe developmental defects that may occur when inactivated by mutation [76, 77, 148]. Cell surface proteoglycans are known to work as binding sites for numerous protein ligands, often acting as co-receptors that promote interaction with conventional receptor proteins [136, 148, 149]. One subclass of proteoglycans that has received particular attention for their receptor roles are the *heparan sulfate proteoglycans (HSPGs)*, containing the GAG heparan sulfate (HS) (Figure 3.4). HS consists of disaccharides units (~ 10-50) built up from *N*-acetylglucosamine (GlcNAc) and glucuronic (GlcA) or in some cases iduronic (IdoA) acid, that are all modified by sulfate groups at different positions. HSPGs are in turn divided into two main families: GPI-anchored *glypicans* and transmembrane *syndecans*. Glypicans have HS chains attached close to the cell membrane and play a crucial role in development and morphogenesis. Syndecans instead display HS chains at their distal ends and function in growth factor regulation and cell adhesion. Certain proteins have binding motifs containing patterns of charged residues that are suggested to promote binding to HS [76, 77]. In addition, HSPGs have been proposed to have a receptor function in uptake of cationic peptides such as CPPs. However, the exact role of HSPGs in CPP uptake is not clear, which is further discussed in section 2.1.2. In biophysical studies of interactions with sulfated sugars, heparin is often used as a mimic for cell surface HS. Heparin and HS differ to some extent in their saccharide units (iduronic acid instead of glucuronic acid in heparin) and degree of sulfation, with heparin being more highly sulfated.

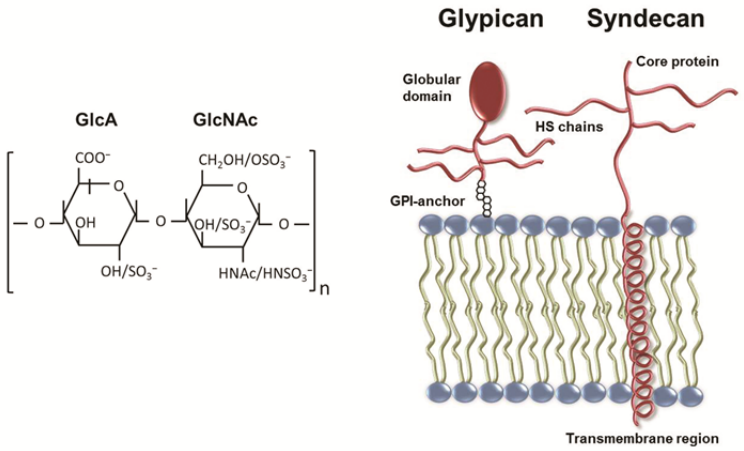


Figure 3.4 Schematic overview of heparan sulfate proteoglycans (HSPGs). Left: Chemical composition. Right: Membrane organization.

3.3.2. Endocytosis

The plasma membrane serves as a selective barrier that efficiently prevents transport of large and polar molecules, whereas small and hydrophobic molecules can permeate the membrane by passive diffusion. However, most molecules bypass direct membrane transport by using highly regulated processes. These are performed by designated membrane transporter proteins that either act as carriers or channels through the membrane, or as receptors for initiation of endocytosis. *Endocytosis* is a general term for

several diverse processes in which the cell instead internalizes macromolecules through the formation of membrane vesicles. As discussed in section 2.1.2., it is also considered to be a major route of entry for CPPs.

During endocytosis, extracellular material becomes enclosed into membrane vesicles called endosomes that pinch off from the plasma membrane on the intracellular side. Once inside the cell, endosomes undergo a maturation process from "early" to "late" endosomes. The maturation is accompanied by acidification, gradually lowering the endosomal pH. If not retrieved from the endosomes, the endocytosed material will end up in lysosomal compartments, the principal sites of intracellular digestion, which are rich in degrading enzymes such as acid hydrolases.

Endocytosis can be divided into two main categories: phagocytosis ("cell eating") or pinocytosis ("cell drinking"). *Phagocytosis* is a receptor-mediated process performed by specialized cells in the immune system and involves engulfment of large particles by actin-driven protrusions. *Pinocytosis*, on the other hand, is performed by essentially all cell types and can occur *via* several different pathways including macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis (Figure 3.5). The pinocytic rate differs between cell types, but typically ~1% of the membrane is ingested each minute [136].

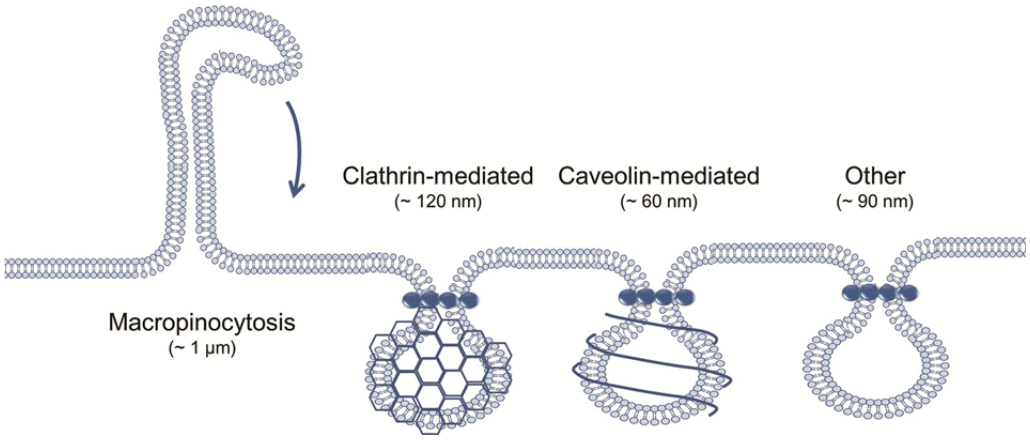


Figure 3.5 Schematic illustration of different pinocytosis mechanisms.

Macropinocytosis differs mechanistically from other endocytic processes in the way the vesicles are formed. Whereas pinocytosis is normally initiated at sites of plasma membrane invagination, macropinocytosis occurs at locations of membrane protrusion, driven by actin polymerization. These protrusions collapse back and fuse with the membrane, encapsulating and internalizing both membrane-bound material and extracellular fluid. The vesicles that are formed are irregular in shape and large (typically 1-5 µm in diameter) compared to other endosomes (~100 nm). Macropinocytosis is most common at specific membrane sites where the cells are continuously creating waves of actin protrusions, a process called *cell surface ruffling*. In most cells, macropinocytosis levels are intrinsically low, but they can be transiently induced by growth factors and other external stimuli. Triggering of macropinocytosis involves activation of receptor tyrosine kinases, resulting in signalling

cascades including the Rho family of GTPases, acting in concert with phosphoinositide 3-kinase (PI(3)K) to initiate actin polymerization and macropinosome closure [150, 151]. While the exact role(s) of macropinocytosis remains somewhat elusive, it has emerged as a key mechanism for delivery of a range of macromolecules [150], including cell-penetrating peptides [152], making this pathway particularly interesting from a drug delivery point of view. In addition, macropinosomes have intriguingly been observed to remain isolated from the endolysosomal system, indicating a distinct pathway with possible potential to escape endosomal degradation [153].

As mentioned above, other endocytic mechanisms involve invaginations of the plasma membrane that eventually bud off to form endosomes. In the case of *clathrin-mediated endocytosis (CME)*, membrane deformation is aided by a protein network and the endosome is pinched off by the GTPase dynamin, which assemble as a ring around the neck of each bud. Clathrin-coated vesicles also provide an efficient uptake pathway for selective receptor-mediated endocytosis, where specific macromolecules bind to cell-surface receptors prior to internalization. *Caveolae-mediated endocytosis* is instead initiated at flask-shaped invaginations in the plasma membrane called caveolae, which are formed at membrane domains rich in cholesterol and sphingolipids [154]. The shape of the caveolae is conferred by the cholesterol-binding protein caveolin that forms a coat on the surface of the membrane invaginations. In addition, a number of less well-defined endocytic mechanisms that are seemingly independent of both clathrin and caveolin also exist. The different endocytic mechanisms are thoroughly described in the review by Conner and Schmid [155] and their importance for CPP internalization is discussed in section 2.1.2.

3.3.3. Model Membranes

From the description of biological membranes above, it stands clear that these are remarkably complex in both structure and composition. Due to difficulties in adapting cellular membranes to most biophysical techniques, more simplified model systems in the form of planar *lipid bilayers* or spherical vesicles called *liposomes* are often used to investigate membrane interactions [156]. Despite that they lack proteins and carbohydrates, these model systems have been enormously valuable in studying lipid interactions at a molecular level. The choice of model system is directly dependent on the type of experiment. While planar bilayers are used with techniques such as Atomic Force Microscopy (AFM), Quartz Crystal Microbalance (QCM), or solid state Nuclear Magnetic Resonance (NMR), vesicles are often used to study interactions in solution by different spectroscopic techniques or by microscopy.

The membrane models described above constitute well-defined systems, making it possible to explore particular interactions in detail. However, the main limitation is that these simplified systems sometimes lack sufficient similarity with actual cell membranes, making it difficult to draw conclusions that are valid in a biological context. This has become a particular concern in CPP research, since it has indeed been difficult to find robust correlations between interactions with model lipid membranes and uptake in live cells [43, 47, 157]. Therefore, in this Thesis, a membrane model that is more representative of the

plasma membrane is explored to assess cell surface association of CPPs as opposed to lipid bilayer interaction. *Plasma Membrane Vesicles (PMVs)* is a novel model system in CPP research, but have previously been used for a number of different applications including studies of membrane phase separation [158], lateral diffusion by membrane proteins [159], lipid-protein interactions [160], membrane protein proteomics [161], and as a membrane source for nanotechnological applications [162].

Development of PMVs is based on a discovery made more than 30 years ago, when it was found that cells can release micrometer-sized vesicles upon exposure to formaldehyde (FA) and dithiothreitol (DTT) [163-165]. Since then, the formation mechanism, often referred to as *blebbing*, has been investigated to some degree and it is believed that these vesicles are formed by weakening of bonds between the plasma membrane and the cytoskeleton, followed by a volume expansion driven by the intracellular pressure [166-168]. Importantly, PMVs formed by the FA/DTT method have been shown to have general characteristics of the plasma membrane, containing all major classes of phospholipids, with some enrichment of sphingomyelin and cholesterol. The interior of the vesicles contains cytosolic fluid, with no evidence of cytoskeletal structures, cytoplasmic organelles or nuclear fragments [163, 164]. An advantage with PMVs, apart from their representative membrane composition and organization, is that they lack the cellular machinery needed for endocytosis, making it possible to specifically study peptide-membrane interactions (as opposed to internalization). In this Thesis, PMVs are adapted for biophysical studies of cell surface binding of CPPs. The possibility to develop these vesicles for an even wider range of biophysical techniques makes PMVs an attractive alternative in between live cells and simpler membrane models.

4. METHODOLOGY

This Chapter presents a short theoretical background to the main experimental techniques used for the work in this Thesis.

4.1. SPECTROSCOPY

The basis of all spectroscopic techniques is the interaction between electromagnetic radiation, *i.e.* light, and matter. Light consists of an electric and a magnetic field oscillating perpendicular to each other and to the direction of propagation. When travelling through space, light behaves like a *wave*, having a certain wavelength, frequency and polarization, but upon interaction with molecules, light is best described as discrete energy packages termed *photons*. Thus, light is characterized by both wave- and particle-like properties, a concept known as *wave-particle duality*. By analysing the absorption, emission or scattering of photons, information about a sample can be obtained at the molecular level. This section describes the spectroscopic techniques used in this Thesis, and the basic theory behind them. More comprehensive reviews on spectroscopy in general, or fluorescence spectroscopy, polarized spectroscopy and light scattering in particular, can be found in textbooks by Hollas [169], Lakowicz [131], Nordén *et al.* [170] and Finsy [171].

4.1.1. Absorption Spectroscopy

Molecules can upon exposure to light absorb photons and thereby undergo a transition to a higher energy level, that is become excited. For this event to occur, the energy of the photon, given by its oscillation frequency (υ), must correspond exactly to the gap between two energetic states in the molecule (ΔE). This criterion is stated by the Bohr frequency condition:

$$\Delta E = E_2 - E_1 = h\upsilon = \frac{hc}{\lambda} \qquad \text{(Equation 4.1)}$$

where h is Planck's constant, c is the speed of light and λ is the wavelength of the absorbed light. In addition, the probability of absorption is dependent on constructive interference between the electric field of light and the transient oscillation of the electrons as the molecule changes its electronic state. The oscillating field induces a dipole moment, $\vec{\mu}_{fi}$, in the molecule given by:

$$\vec{\mu}_{fi} = \int \psi_f \, \mu \, \psi_i \, d\tau \qquad \text{(Equation 4.2)}$$

where μ is the electric dipole operator and Ψ_i and Ψ_f the wavefuntions of the initial and final states respectively. $\vec{\mu}_{fi}$ can be regarded as an "antenna" that must be aligned with the electric field vector of light for maximum probability of absorption. The absorption, A, is thus proportional not only to the magnitude of $\vec{\mu}_{fi}$, but also to the orientation of $\vec{\mu}_{fi}$ relative to the incident light:

$$A \propto \left|\vec{\mu}_{fi}\right|^2 \cos^2 \theta \qquad \text{(Equation 4.3)}$$

where θ is the angle between $\vec{\mu}_{fi}$ and the electric field vector.

The absorbance, A, can be calculated from the incident and transmitted light intensities (I_0 and I respectively) as light is passed through a sample, and depend on sample path length *(l)* and concentration of absorbing molecule *(c)* according to the Beer-Lambert Law:

$$A = \log \frac{I_0}{I} = \varepsilon(\lambda) c \, l \qquad \text{(Equation 4.4)}$$

where $\varepsilon(\lambda)$ is the molar extinction coefficient, which is related to the oscillator strength of the transition in the molecule. The linear relation between absorbance and concentration makes absorption spectroscopy an important technique for concentration determinations, *i.e.* as a quantitative tool, and is used as such also in this Thesis. However, absorption may also be used to obtain more detailed information on molecular geometry or interaction.

4.1.2. Fluorescence Spectroscopy

Following the process of absorption, a molecule will eventually relax back to its ground state by dissipation of the excitation energy. This deactivation process can occur though both non-radiative and radiative pathways, the latter involving emission of a photon. Depending on the nature of the excited state, emission is in turn divided into fluorescence or phosphorescence. *Fluorescence* is emission from a singlet state, S_n, where the excited electron retains its spin from the ground state, making the transition spin-allowed and thus rapid. *Phosphorescence* is instead emission from a triplet state, T_n, meaning that it involves a quantum mechanically forbidden spin change, making it considerably slower than fluorescence. The excited state lifetime, τ, is the average time between excitation and return to the ground state and is typically 10 ns for fluorescence and milliseconds to seconds for phosphorescence.

The photophysical processes that occur in a molecule upon absorption of light are depicted in the Jabłoński diagram shown in Figure 4.1. The first event following excitation from the ground state (S_0) to a higher electronic state (S_n), is the relaxation to the lowest vibrational state of S_1 by non-radiative vibrational relaxation *(VR)* and internal conversion *(IC)*. From this state, there are three options for return to the ground state: (1) continued relaxation through non-radiative processes, (2) fluorescence emission or (3) spin conversion

(intersystem crossing, *ISC*) to a triplet state followed by non-radiative relaxation or phosphorescence emission. Due to the relaxation processes that precede emission, the energy of the emitted light is always lower in energy than the absorbed light. This red-shift in emission spectra is referred to as the Stoke's shift. Also, since energy relaxation through emission occurs in parallel with the non-radiative processes, the efficiency of fluorescence emission is expressed in terms of the quantum yield (ϕ_f) and depends on the rate-constants of all deactivation pathways according to:

$$\phi_f = \frac{photons\ emitted}{photons\ absorbed} = \frac{k_f}{\sum_i k_i} \qquad \text{(Equation 4.5)}$$

with k_f being the rate constant for fluorescence emission and $\sum_i k_i$ the sum of all rates of excited state decay. The intensity of fluorescence can also be reduced by a variety of quenching processes, *e.g.* collision with other molecules present in the sample or formation of non-fluorescent complexes. Although quenching can be used as a tool to investigate *e.g.* binding interactions or solvent accessibility, it can also be a complication when measuring fluorescence. In this Thesis, fluorescence is like absorption used as a quantitative tool, with the advantage that fluorescence can be measured in a wider concentration regime than absorption, and quenching is further used to investigate peptide interactions. Fluorescence is also utilized in techniques such as microscopy and flow cytometry to study fluorescently labelled peptides.

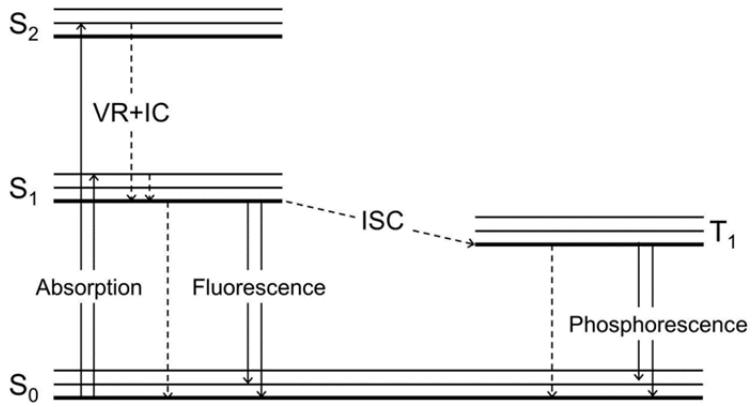


Figure 4.1 A Jabłoński diagram illustrating deactivation pathways following electronic excitation. Solid arrows indicate radiative processes and dashed arrows indicate non-radiative processes.

4.1.3. Polarized Spectroscopy

In conventional absorption spectroscopy, both the incident light and the molecules in the sample are isotropic, *i.e.* without any preferred direction. In polarized spectroscopy, the use of polarized light, and in some case also oriented samples, makes it possible to obtain more detailed information on molecular structure, orientation and symmetry. The techniques are based on the differential absorption of light having different orientation.

Linear Dichroism

Linear Dichroism *(LD)* is the differential absorption of light polarized parallel *(II)* and perpendicular *(⊥)* to a macroscopic orientation axis:

$$LD = A_{II} - A_{\perp} \qquad \text{(Equation 4.6)}$$

In an isotropic sample, the random molecular orientations cancel out the LD signal to zero and in order to give rise to non-zero LD, the sample therefore needs to be anisotropic, *i.e.* macroscopically oriented. This orientation can be achieved in different ways, *e.g.* using stretched polymer films, electric fields, or, as used in this Thesis, a flow gradient. The principle of flow LD is schematically shown in Figure 4.2. The sample is confined to a narrow gap between two concentric quartz cylinders in a so-called Couette cell. Rotation of one of the cylinders creates a laminar shear flow that aligns the molecules in the flow direction. However, in order to align properly, the molecules need to be of a certain length and for DNA that is used in this Thesis, the lower limit is around 1000 basepairs [172]. In DNA, the absorption, and hence the LD signal, is dominated by the $\pi \rightarrow \pi^*$ transitions of the bases that are oriented perpendicular to the DNA backbone and consequently also to the direction of orientation. Therefore, flow-oriented DNA shows a negative LD peak around 260 nm.

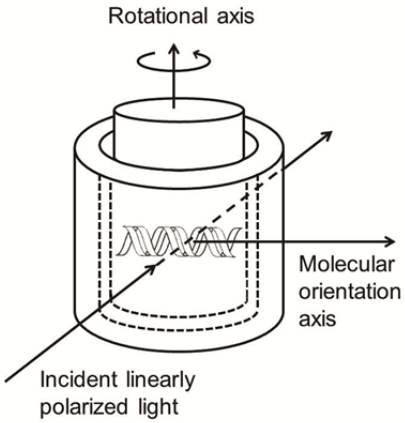


Figure 4.2 The principles of flow LD using a Couette cell. The molecules in the sample are aligned in the shear flow that is created when one of the cylinders is rotated.

Linear Dichroism is a useful tool to determine molecular orientation, which can be done by normalizing the LD signal with respect to the isotropic absorption of the sample *(A_{iso})*. This quotient is referred to as the reduced LD, or *LD^r*, and is related to the angle α between the transition dipole moment and the macroscopic orientation axis according to:

$$LD^r = \frac{LD}{A_{iso}} = \frac{3}{2} S(3\cos^2 \alpha - 1) \qquad \text{(Equation 4.7)}$$

where *S* is the orientation factor that indicate the degree of orientation in the sample and equals 1 for a perfectly oriented sample and 0 for an isotropic sample. Given that the direction of the transition dipole moment in the molecule is known, LD can be used to

deduce the orientation of the molecule relative to the macroscopic orientation axis, *e.g.* the orientation of a DNA-binding ligand relative to the DNA helical axis or the orientation of peptides and proteins in a lipid membrane. LD can also be used to monitor changes in macromolecular structure that leads to a decrease in orientation, *e.g.* condensation of DNA which is studied in this Thesis.

Circular Dichroism

Circular Dichroism *(CD)* is the differential absorption between left *(l)* and right *(r)* circularly polarized light:

$$CD = A_l - A_r \qquad \text{(Equation 4.8)}$$

In order to exhibit CD, the molecules must either be intrinsically chiral or be perturbed in a chiral environment. A CD signal arises when light induces a helical perturbation of charge density. Analogous to the linear charge redistribution (μ_{fi}) induced by the electric field component of light, a circular rearrangement of electrons can be induced by the magnetic field component and this is described by the magnetic dipole moment, m_{fi}. The origin of a CD signal in chiral molecules depends on μ_{fi} and m_{fi} being non-orthogonal, thereby resulting in a net charge rearrangement in the form of a helix. CD is an important tool to study secondary structures of chiral molecules such as DNA and proteins.

4.1.4. Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a technique that is primarily used to measure the size of particles typically in the submicron region. In a DLS measurement, a dilute sample is illuminated by a laser with a wavelength that is not absorbed by the particles in the sample. Most of the laser beam passes through, but a small fraction of the light is scattered in different directions. A detector measures the scattered light at a fixed angle and the angular dependence of the intensity *I(q)* is usually expressed in terms of the scattering vector $|\vec{q}|$:

$$|\vec{q}| = \frac{4\pi n}{\lambda}\sin\frac{\theta}{2} \qquad \text{(Equation 4.9)}$$

where n is the refractive index of the sample, λ is the wavelength of the incident light and θ the scattering angle. The scattered light intensity will fluctuate as the particle moves at a rate that is proportional to the random Brownian motion, and hence to the size of the particles in the sample. The fluctuating intensity is used to construct a second order autocorrelation function ($g^{(2)}(\tau)$), comparing the average intensities ($\langle I \rangle$) at time points t and $\tau = t+\delta t$:

$$g^{(2)}(\tau) = \frac{\langle I(t)I(\tau)\rangle}{\langle I(t)\rangle^2} \qquad \text{(Equation 4.10)}$$

At short time delays $I(\tau) \approx I(t)$, since the positions of the particles are virtually unchanged and hence, the correlation is high ($g^{(2)}(\tau = 0) = 1$). As the time delay increases, the particles

will move further away from their original positions until there is no correlation between the scattered intensity of the initial and final states. As this happens, the correlation curve will decay exponentially (Figure 4.3). For spherical particles, this decay is described by:

$$g^{(2)}(\tau) = A\left[1 + Be^{-2q^2D\tau}\right] \qquad \text{(Equation 4.11)}$$

where A and B are correction factors depending on the experimental setup, q is the previously mentioned scattering vector and D the translational diffusion coefficient of the particles in the sample. The latter can be determined from the autocorrelation function by fitting either a single-, or in the case of polydisperse samples, a multiexponential to the correlation function (Eq. 4.11). If the particles are assumed to be spherical, the translational diffusion coefficient *(D)*, is related to the hydrodynamic radius *(R_H)*, according to the Stokes-Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta D} \qquad \text{(Equation 4.12)}$$

where k_B is Boltzmann's constant, T the absolute temperature and η the viscosity of the solvent. In this Thesis DLS is used to estimate the size of complexes formed between CPPs and either DNA or heparin.

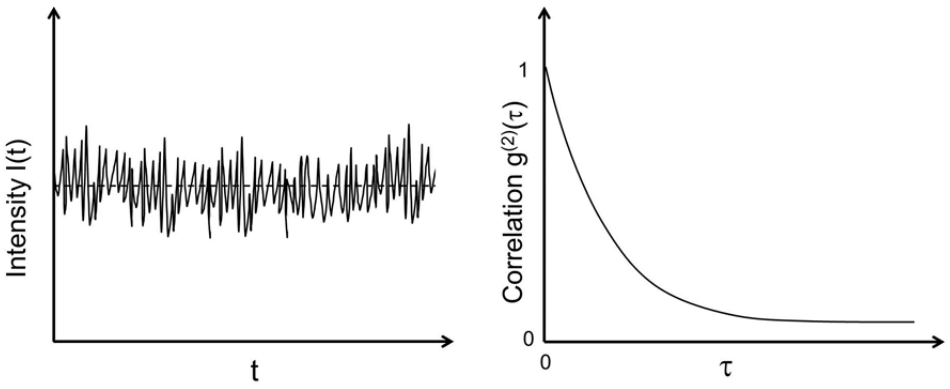


Figure 4.3 Principles of DLS. Left: The intensity variation of the scattered light. Right: The decay of the autocorrelation function.

4.2. MICROSCOPY

Microscopy comprises a wide range of techniques, all used to visualize objects that are too small to be detected by the naked eye. Optical microscopy is, like spectroscopy, based on the interaction between light and matter and a magnified image is obtained by passing light that is transmitted or reflected by the sample through an objective lens. The image resolution *(d)* determines the smallest distance by which two objects are perceived as separate units and in general, the radiation used to visualize a sample cannot resolve details that are considerably smaller than its own wavelength. The second factor determining the image resolution is the numerical aperture of the objective, *NA*, which characterizes the

ability of the objective to collect light. This limitation in image resolution is defined in the Rayleigh criterion:

$$d = 0.61\frac{\lambda}{NA} \qquad \text{(Equation 4.13)}$$

NA is in turn determined by the refractive index (n) of the immersion medium between the objective and the specimen (usually water, n = 1.33 or oil, n = 1.52) and the half-angle of the maximum cone of light that can enter the objective θ, according to:

$$NA = n\sin\theta \qquad \text{(Equation 4.14)}$$

The resolution of an optical microscope is typically 0.2-0.5 μm, although under optimal conditions, using ultraviolet (UV) light sources and objectives with high numerical apertures (NA = 1.4), the resolution limit is about 150 nm. Eukaryotic cells that are studied in this Thesis are typically 10 μm in diameter and can thus be visualized with good intracellular detail.

The foundation of optical microscopy described above was established more than a century ago [173]. However, the introduction of more modern techniques such as laser light sources, electronic image detection and advanced image analysis, have opened up for new applications. In addition, development on the sample preparation side today makes it possible to image live cells, stained with highly specific fluorescent markers.

4.2.1. Fluorescence Microscopy

Fluorescence microscopy is a widely used method in biological sciences, due to the possibility to specifically stain and visualize cellular structures that are invisible in normal light microscopy. In principle, the sample is illuminated by a specific wavelength that matches the excitation spectrum of the fluorescent dye. This can be done either using a lamp with an appropriate wavelength filter, or using a laser source that can provide a more coherent beam with several orders of magnitude higher intensity. The radiation excites the fluorophore and detects the red-shifted emitted light through a filter that excludes the excitation wavelength. By using multiple excitation wavelengths and/or emission filters, several fluorophores can also be detected simultaneously, allowing for example co-localization to be studied.

4.2.2. Confocal Laser Scanning Microscopy

In conventional fluorescence microscopy, the whole sample is illuminated at all times. This generates fluorescence also from optical sections that are out of focus, limiting spatial resolution. To avoid this problem, confocal optics can be used. The basic principle is rather simple and involves focusing the light at any instance on a single point at a specific depth of the sample. This is often accomplished by the use of two pinholes; one to achieve focused point illumination and another to exclude out-of-focus emission from reaching the detector. The configuration of the two pinholes are said to be *confocal*, *i.e.* placed at positions so that the emission from the in-focus point come to a focus just at the detector

pinhole. In Confocal Laser Scanning Microscopy (CLSM), the laser scans the sample across the plane of focus and builds up a 2D image, and by scanning several planes consecutively, detailed 3D images can also be reconstructed. This technique is well suited for thick, scattering samples such as biological tissues and elimination of out-of focus light also leads to less sample damage in terms of fluorophore photobleaching, heat-induced damage and phototoxicity to live cells. However, the pinholes also result in one major drawback, and that is the limited light that reaches the detector. This means that several scans are often required to obtain enough information which in turn reduces the speed of acquisition. This is a limitation in studies of fast dynamic events, but technical advances such as spinning disks can reduce the acquisition time down to milliseconds. A comprehensive review of confocal microscopy in biological sciences is found in the textbook by Pawley [173]. In this Thesis, CLSM is used to study plasma membrane binding, cellular uptake and intracellular localization of fluorescently labelled CPPs.

4.3. FLOW CYTOMETRY

Flow cytometry combines the principles of light scattering and fluorescence to obtain multiparameter information from a sample of microscopic particles such as cells. By combining a high throughput with individual analysis of each cell, statistical data can be obtained for large cell populations.

The basic principles and applications of flow cytometry are thoroughly described in several textbooks [174-176]. In short, a beam of laser light is directed onto a stream of cells suspended in aqueous solution. For accurate data collection, it is important that only one cell at a time passes through the laser light. In most flow cytometers, this is accomplished by *hydrodynamic focusing*, *i.e.* the cell suspension is injected into a flow stream of sheath fluid and the sample flow thereby becomes compressed to approximately one cell in diameter. An alternative approach is to use a microcapillary system, with a capacity to focus single cells without the use of sheath fluid [177].

As a cell passes through the laser beam, it will scatter light in all directions and the instrument detects both forward scatter, which is proportional to the size of the cell, and side scatter at 90° angle, which is proportional to the granularity and structural complexity inside the cell. Usually, 2D scatter plots (Figure 4.4) is constructed from the forward and side scatter and cell samples can thus be characterized and divided into different subpopulations based on these two parameters. Additional information can be achieved by the use of fluorescent labelling and by using multiple excitation lasers and/or emission filters, several fluorophores can be detected simultaneously. By constructing scatter plots, single or double labelling of different fluorescent markers can be analysed and combined with the light scattering data, this allows for detailed analysis of complex biological systems. In this Thesis, flow cytometry is used to quantify plasma membrane binding and uptake of fluorescently labelled peptides as well as peptide-mediated transfection using *Enhanced Green Fluorescent Protein (eGFP)* as reporter protein. eGFP is a variant of the native GFP protein [178] that contains chromophore mutations to enhance brightness, and is codon-optimized for higher expression in mammalian cells [179].

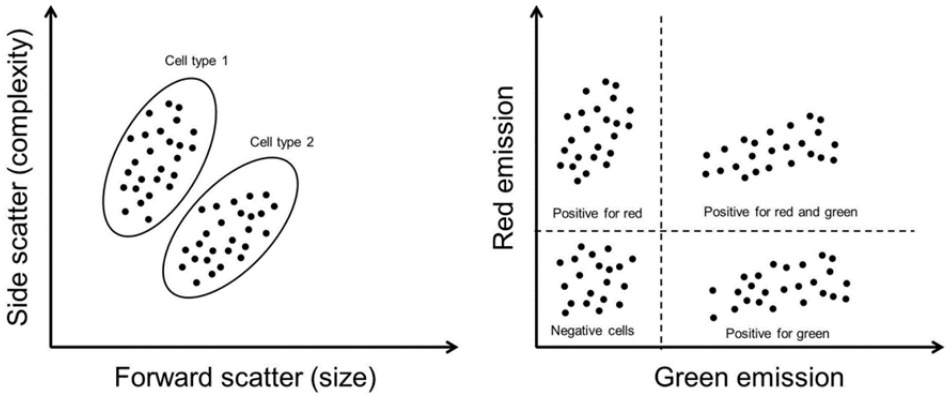


Figure 4.4 Analysis of flow cytometry data using scatter plots. The cell population is characterized based on light scattering (left) and emission (right).

4.4. GEL ELECTROPHORESIS

Gel electrophoresis is a common method in molecular biology used for separation of biological macromolecules such as DNA and proteins, for both analytical and preparatory purposes [135, 175, 176]. The technique is based on the electrophoretic motion of charged molecules in an electric field. By applying an external electric field on a sample, charged species will move towards the oppositely charged electrode, *i.e.* cationic molecules towards the cathode and anionic towards the anode. This motion can be described by the electrophoretic mobility, μ, which is determined by the ratio between the speed of the molecule, v, and the electric field strength, E, as well as by the ratio between the charge of the molecule, q, and the friction coefficient in the medium, f:

$$\mu = \frac{v}{E} = \frac{q}{f} \qquad \text{(Equation 4.15)}$$

The friction coefficient f is in turn influenced by both *size* and *shape* of the molecule and these factors together with *charge* thus determine the mobility. In principle, separation can be achieved based on either of these factors, but separation by size is most common. However, for a polymer with uniform charge distribution, such as DNA, the electrical and frictional forces acting on each monomer unit would cancel each other out when moving free in solution, rendering the mobility independent of molecular size. To achieve separation by molecular size, the sample is therefore placed in a polymer gel and depending on the type and concentration of polymer, different pore sizes can be obtained. The two most common polymers used are agarose, which is a linear polysaccharide, and polyacrylamide, which is obtained by polymerizing and crosslinking acrylamide monomers. Due to the restricted movement in the polymer matrix, a size-, but also shape-dependent separation will take place, resulting in distinct bands that can be visualized by staining with a fluorescent dye. By comparing the pattern of bands to known standards, the contents of the sample can be determined empirically. In order to avoid influence from shape, the sample can be denaturated prior to gel electrophoresis, but the shape dependence can also be exploited to distinguish between different configurations, such as linear, circular and

supercoiled plasmid DNA. For proteins, which are more diverse than DNA in terms of charge and shape, separation according to size is always performed under denaturating conditions by treatment with the anionic detergent sodium dodecyl sulfate (SDS) in combination with a reducing agent to break disulfide bridges. In addition to denaturation, SDS also creates a uniform charge distribution and thus enables separation by size.

Gel electrophoresis is a common technique to study both DNA and proteins, but can actually be used also to probe the interaction between them. Gel retardation is a widely used method to investigate protein-nucleic acid interactions [180] and can be used also in the study of DNA condensation. However, the concentrations employed in gel electrophoresis often result in intermolecular aggregates larger than the ~100 nm pore size of the gel [181], resulting in difficulties to even enter the gel. Exclusion from the gel can thus be a measure of DNA condensing capacity. More advanced applications include stability assays, such as the one used in this Thesis, where the amount of intact DNA is assessed following exposure to nucleases and dissociation of DNA before loading onto a gel.

4.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) is a widely used technique for separation of individual components in liquids and can be employed either for sample purification or as an analytical tool to study *e.g.* binding affinity [175, 176, 182].

The basis of all types of chromatography is the differential partitioning of compounds between a stationary solid phase and a liquid mobile phase. The stationary phase is most commonly attached to a suitable matrix (an inert, insoluble support) and packed into a column and the mobile phase, commonly referred to as the eluent, is passed through the column. In HPLC, a small particle size in the stationary phase is used, which enhances the chromatogram resolution by increasing the surface area, but also results in dense packaging. Hence, a pump (rather than gravity) is used to provide the high pressure needed to move the mobile phase through the column. A detector, such as a UV/Vis spectrometer, records the retention time(s) of the compound(s) in the eluent. The analyte retention time is dependent on the strength of interaction with the stationary phase, the composition of solvents and the flow rate through the column. Separation can either be performed with a constant composition of the eluent, called isocratic elution, or by gradually changing the eluent composition in a so-called gradient elution. In this Thesis, the technique is used to assess binding affinity of CPPs towards heparin, by performing a gradient elution that gradually weakens the intramolecular forces.

5. RESULTS

In this Chapter, the most important results from Papers I-V are summarized and discussed. Some relevant unpublished results are also presented.

The work presented in this Thesis focuses on the well-known CPP *penetratin* and two analogs named *PenArg* and *PenLys* that have previously been designed in our lab to study the relative importance of arginine and lysine for CPP properties and function [38, 43, 47, 68]. This triplet of peptides were assessed in terms of their uptake efficiency and uptake mechanism (*Paper I*), their relative affinity for the cell surface (*Paper II*), their dependence on cell surface proteoglycans for membrane association and uptake (*Paper III*), and finally their capacity to form peptiplexes and deliver plasmid DNA into cells (*Papers IV-V*).

5.1. UPTAKE OF PENETRATIN PEPTIDES

The ability to internalize into cells is naturally a key property for a cell-penetrating peptide. Uptake from the extracellular environment into the cell can be considered as a two-step process involving (1) binding to the cell surface and (2) transport to the cell interior. Hence, the capacity of a CPP to internalize into cells should intuitively be governed by its cell surface affinity, either on its own or in combination with the ability to stimulate uptake at the cell surface. These questions were addressed in the work of this Thesis.

In *Paper I*, the capacity of penetratin peptides to internalize into cells by triggering the cell's endocytic activity was explored. The incentives behind this work came from two previous studies by Thorén *et al.* [38] and Persson *et al.* [47]. In the first of these studies, Thorén *et al.* studied cellular uptake by confocal microscopy and accentuated arginine-dependent differences in the capacity to internalize into cells was indicated, with penetratin being internalized *via* endocytosis, PenArg *via* both endocytosis and direct penetration, while PenLys was seemingly unable to enter cells at all. This prompted us in *Paper I* to explore uptake in a quantitative manner and also to look further into the uptake mechanism. In the second study mentioned above, Persson *et al.* tried to explain the diverging uptake capacities of PenArg, penetratin and PenLys in terms of differences in membrane affinity, but found only a 3-fold difference in surface partition constants for anionic lipid membranes. This pointed towards that membrane affinity was not decisive for uptake, and we therefore decided to instead search for differences in the capacity to trigger an uptake response. Therefore, in *Paper I*, the ability of the penetratin peptides to stimulate

macropinocytosis was assessed, as it had recently been found to be involved in uptake of arginine-rich peptides such as Tat [144, 183] and oligoarginines [82, 91].

Cellular uptake of fluoresein-labelled penetratin peptides was studied using both a quantitative uptake protocol to directly compare their uptake efficiencies, and confocal microscopy to assess their intracellular localization. Figure 5.1A shows the relative uptake of penetratin peptides in Chinese hamster ovarian cells (CHO-K1) after 1 h incubation. To ensure complete removal of extracellularly bound peptide, the cells were washed with heparin (100 μg/ml, which is 10 times the concentration needed to completely inhibit cell surface binding of the CPP Tat [144]), prior to trypsination and subsequent cell lysis. In the investigated concentration regime (1-10 μM), the uptake levels are nearly linearly dependent on concentration and are clearly in the order PenArg > penetratin > PenLys. PenArg is roughly 10 times more efficiently internalized than PenLys. These results show that arginines are beneficial, but not a prerequisite, for uptake and that substituting arginines for lysines leads to a gradual decrease in uptake, but not complete inhibition, which had been previously suggested [38].

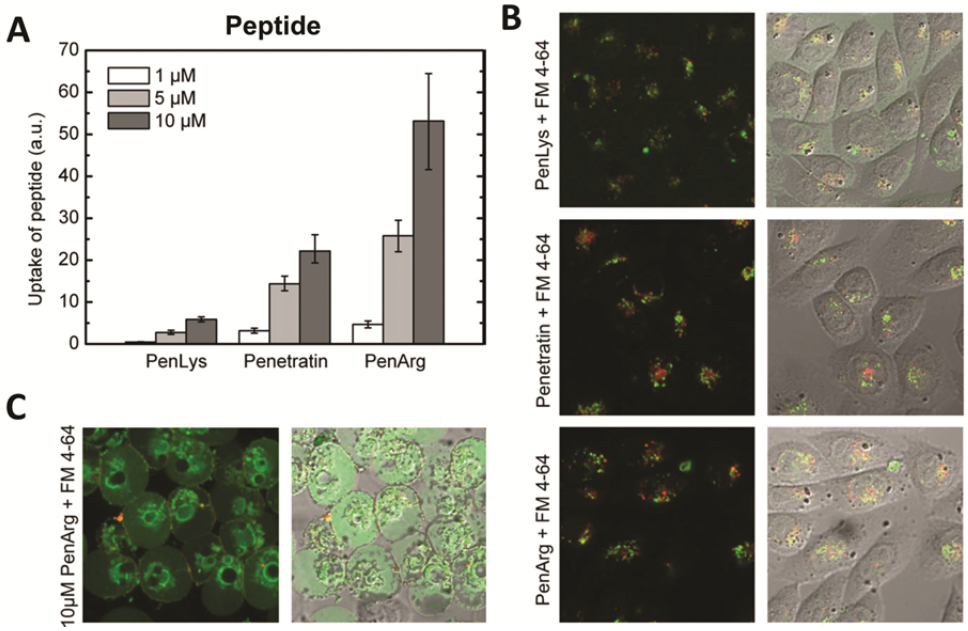


Figure 5.1 Uptake of penetratin peptides in CHO-K1. **(A)** Peptide uptake after 1 h incubation at 37°C as a function of peptide concentration. The bars represent the average of nine independent experiments, performed in triplicate (N=9, n=3). Error bars indicate the average maximum and minimum deviation in each experiment. **(B)** Confocal images of live CHO-K1 cells showing uptake of FITC-labelled peptide (5 μM, green) and its co-localization with the non-specific endocytosis marker FM4-64 (5 μg/ml, red) after 1 h incubation at 37°C. The image contrast and detector gain was optimized for each micrograph and the intensities in each image are thus not comparable. **(C)** Uptake and distribution of PenArg applied at 10 μM concentration showing compromised cell morphology.

Peptide internalization was also studied using confocal microscopy and co-incubation with FM4-64, which is a general marker for endocytosis. FM4-64 is a lipophilic styryl dye that is virtually non-fluorescent in aqueous media but becomes intensely fluorescent once inserted into the membrane. It is unable to translocate the membrane directly but is internalized

into endocytic vesicles. Figure 5.1B shows confocal images of CHO-K1 cells incubated with 5 μM of penetratin peptides and FM4-64 for 1 h. The punctuate intracellular distribution and substantial co-localization with FM4-64 verifies mainly endocytic uptake for all three peptides. No diffuse cytosolic staining (which would suggest direct penetration or highly efficient endosomal escape) was observed except occasionally for PenArg at the highest peptide concentration (10 μM, Figure 5.1C). However, diffuse cytoplasmic staining was in this case accompanied by severely compromised cell morphology, which either means that direct penetration leads to toxicity or the opposite; that membrane-mediated toxicity results in peptide penetration.

5.1.1. Stimulation of Endocytosis

The ability of the penetratin peptides to stimulate macropinocytosis was assessed by co-incubating peptide with markers of endocytosis. A high molecular weight (70 kDa) neutral dextran labelled with Texas Red (TR-dextran) was used to specifically probe macropinocytosis [184]. As reference, the overall endocytic activity was determined by the uptake of FM4-64. We wanted to explore macropinocytic stimulation in a quantitative manner and Figure 5.2A-B shows the extent of TR-dextran and FM4-64 uptake respectively, both in the absence (control) and presence of penetratin peptides. Addition of peptide markedly increases internalization of TR-dextran to a degree that is dependent on both peptide concentration and arginine content, and thus in qualitative agreement with peptide internalization. By contrast, uptake of FM4-64, end hence the overall endocytic activity, is essentially unaffected by peptide addition except at 10 μM of PenArg. We believe that due to the toxicity observed for PenArg at this concentration, internalization of FM4-64 is most likely exaggerated as a consequence of increased membrane permeation. Uptake of TR-dextran was also studied by confocal microscopy and from Figure 5.2C, it is evident that addition of peptide increases the uptake of TR-dextran. Co-localization between peptide and TR-dextran is however not perfect, which means that the penetratin peptides are not exclusively internalized *via* one endocytic pathway. This is also in agreement with previous observations that several mechanisms are involved in penetratin uptake [55].

The main conclusion from *Paper I* was that the penetratin peptides can trigger their own uptake by specifically stimulating macropinocytosis. Since the overall endocytic activity remains largely constant, this macropinocytic up-regulation occurs at the expense of other endocytic pathways. Moreover, arginine- and lysine-rich penetratin versions have at least some internalization mechanism in common, although lysines are less efficient. This demonstrates that events leading to stimulation of endocytosis are not dependent on specific interactions with arginine sidechains. Still, arginines clearly evoke a higher response than lysines, which means that arginines either have higher affinity for uptake-triggering membrane sites, or that arginines can form specific interactions with additional binding sites and trigger uptake *via* pathways that are not accessible to lysines. These questions were addressed in *Papers II-III*, where the importance of cell surface affinity *versus* uptake stimulation for internalization of arginine- and lysine-rich penetratin versions was explored (sections 5.2.-5.4.).

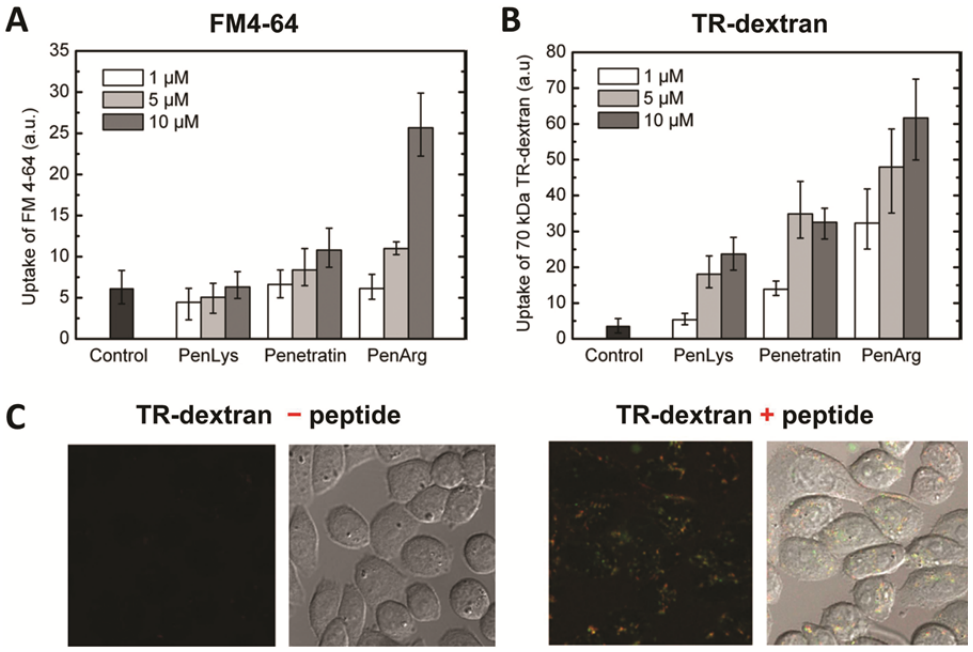


Figure 5.2 Peptide-induced stimulation of macropinocytosis in CHO-K1. (A) Internalization of the non-specific endocytosis marker FM4-64 (5 µg/ml) in absence (control) and presence of penetratin peptides (PenLys, penetratin and PenArg). **(B)** Internalization of macropinocytic marker TR-dextran (100 µg/ml) in absence (control) and presence of penetratin peptides. **(C)** Uptake of TR-dextran (50 µg/ml) in absence and presence of peptide (5 µM PenLys). In all experiments, the cells were incubated for 1 at 37°C. The bars in (A) and (B) represents the average of three independent experiments, performed in triplicate (N=3, n=3). Error bars represent the average maximum and minimum deviation in each experiment.

To obtain further insight into the mechanism by which penetratin peptides trigger macropinocytic up-regulation, we decided to explore known stimulators of macropinocytosis. Transient induction of cell surface ruffling and macropinocytosis can for example be obtained by growth factor stimulation and one of the most well-known stimulators of macropinocytosis is the *epidermal growth factor (EGF)* [185]. EGF is a 53 a.a. peptide that induces membrane ruffling by binding to the EGF receptor, which then triggers activation of receptor tyrosine kinases that in turn leads to intracellular signalling cascades [151]. We decided to explore macropinocytic stimulation of penetratin peptides using EGF as a reference, and unpublished data from these studies are presented below (Figure 5.3 and 5.4).

First, since EGF is known to stimulate macropinocytosis already at nanomolar concentration [185], the capacity of the penetratin peptides to evoke a response in this low, but biologically relevant, concentration regime was investigated. Figure 5.3 shows confocal images of CHO-K1 cells incubated with TR-dextran and 2 nM of EGF, PenArg, penetratin or PenLys for 1 h. This shows that PenArg and penetratin have a stimulatory capacity that is comparable to EGF, while PenLys is less efficient. The fact that these peptides are such competent stimulators of macropinocytosis is intriguing. However, since EGF binding to the EGF receptor is known to involve specific interactions with three large domains in the EGF sequence [186], it is unlikely that the penetratin peptides can stimulate macropinocytosis through this receptor. Instead, it seems as if signalling cascades that

activate macropinocytosis can be initiated by binding of penetratin to additional cell surface binding sites. The nature of these sites and the mechanism by which penetratin can trigger macropinocytosis remains to be identified, but one hypothesis suggested in the literature is that clustering of proteoglycan chains by cationic peptides could trigger endocytosis [76, 77].

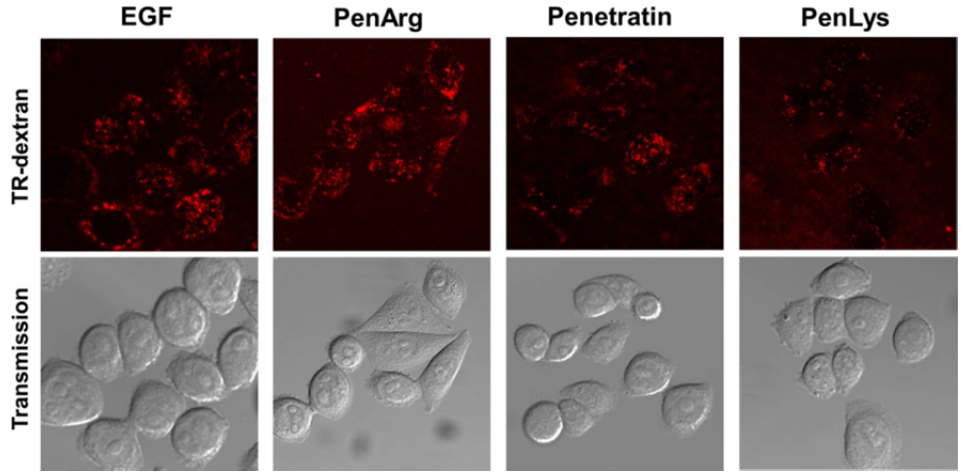


Figure 5.3 Stimulation of macropinocytosis at nanomolar peptide concentrations.
Internalization of macropinocytic marker TR-dextran (50 μg/ml) in presence of 2 nM epidermal growth factor (EGF) or penetratin peptides (PenArg, penetratin, PenLys) after incubation for 1 h at 37°C.

Another question that was raised during the work presented in *Paper I* was whether the penetratin peptides would only stimulate their own uptake at the site where they were bound, or if signalling cascades could be initiated that would enhance macropinocytic activity across the entire cell, thereby also increasing uptake of other molecules. In the latter case, it should be possible to improve uptake of a poorly internalized CPP by addition of another peptide with a strong capacity to stimulate macropinocytosis. To address this question, the ability of unlabelled peptide, applied at nanomolar concentration, to enhance uptake of another fluorescein-labelled peptide applied in micromolar concentration, was investigated. Figure 5.4 shows uptake of 5 μM fluorescein-labelled PenLys (F-PenLys) in absence (control) or presence of three different concentrations of either PenLys itself, PenArg or EGF (as reference). With 5 nM unlabelled peptide added simultaneously to 5 μM F-PenLys, EGF enhances F-PenLys uptake by approximately 20%, which is similar to what has previously been observed for octaarginine [91], and gives further support to the involvement of macropinocytosis in PenLys uptake. In contrast, PenLys and PenArg only have a marginal effect with less than 10% difference from F-PenLys alone and PenArg even show a tendency to inhibit F-PenLys uptake. These results show that whereas EGF has some capacity to enhance F-PenLys uptake by increasing the overall macropinocytic activity, the penetratin peptides appear to stimulate uptake specifically at the site where they bind.

To investigate whether a larger stimulatory effect could be achieved, the concentration of unlabelled peptide was increased to 50 and 500 nM, and in addition the cells were pre-incubated with peptide for 1 h before addition of 5 μM F-PenLys, in order to trigger macropinocytic activity in advance. However, instead of obtaining a stimulatory effect, uptake of F-PenLys was inhibited under these conditions. Interestingly, pre-incubation with EGF was strongly inhibiting and it is possible that EGF at these elevated concentrations exhausts the cell's endocytic machinery during the pre-incubation step and thereby prohibit subsequent F-PenLys uptake. It may also be that EGF binds, and thereby blocks, PenLys binding sites when specific receptor binding becomes saturated. Pre-incubation with PenLys and PenArg results in concentration- and arginine- dependent inhibition of F-PenLys uptake. This might be caused by saturation of peptide uptake upon prolonged incubation, which is supported by observations that uptake of penetratin peptides level off after 1 h incubation (see Figure 1 of *Paper III*). An alternative explanation is that unlabelled peptide during the co-incubation competes for uptake-promoting cell surface binding sites and that PenArg thereby outcompetes F-PenLys. This would in turn mean that peptide affinity for the cell surface is decisive for uptake, a topic which was also addressed in *Paper II*.

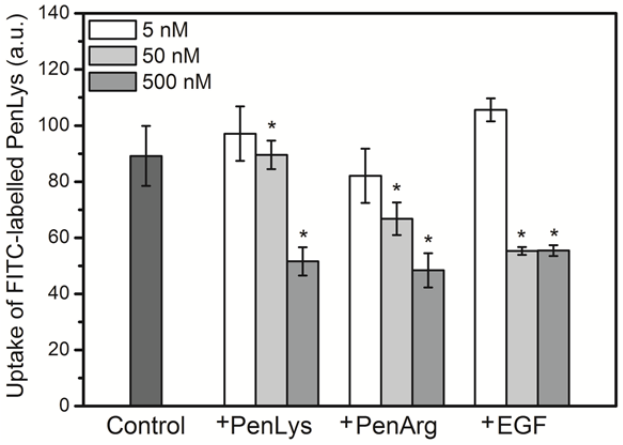


Figure 5.4 PenLys uptake cannot be enhanced by macropinocytic stimulation by other peptides. Uptake of 5 μM fluorescein FITC-labelled PenLys after 1 h incubation at 37°C in absence (control) or presence of 5, 50 or 500 nM unlabelled PenLys, PenArg or epidermal growth factor (EGF). The data represent mean ± S.D. from 3 samples. *Unlabelled peptide was added 1 h prior to addition of FITC-PenLys.

The conclusion from these experiments was that penetratin peptides efficiently stimulate macropinocytosis and, like EGF, evoke this biological response already at nanomolar concentrations. The stimulation appears however local and occurs specifically at the site where the peptide binds. On the one hand, this means that these peptides are capable of stimulating their own internalization, but cannot be used to enhance uptake of other therapeutic molecules. On the other hand, this may also be advantageous as unspecific internalization of unwanted molecules upon treatment with CPPs could increase the risk of side-effects.

5.2. CELL SURFACE BINDING OF PENETRATIN PEPTIDES

Binding to the cell surface is the first step towards internalization of CPPs and it is also the focus of *Paper II* in this Thesis. Although it seem intuitive that the membrane binding affinity of a particular CPP should be important for its corresponding uptake efficiency, some reports in the literature suggest that no such correlation exists [88, 187] and previous work from our group has revealed only weak connections between uptake and peptide-lipid interactions [43, 47]. This suggests that uptake could be primarily dependent on the peptide's capacity to evoke a biological response that triggers internalization, as was observed in *Paper I*, and to a lesser extent on the amount of peptide that resides on the cell surface. However, the lack of correlation between model membrane binding and uptake could also depend on that the lipid vesicles are too simplified to accurately describe peptide-cell surface interactions. In *Paper II*, we therefore explored the influence of arginine and lysine on cell surface binding of penetratin in a more complex model. This was assessed using plasma membrane vesicles (PMVs) released directly from CHO-K1 cells in response to treatment with formaldehyde (FA) and dithiothreitol (DTT) (Figure 5.5). PMVs, which are more thoroughly described in section 3.3.3., have previously been used for a number of applications [158-162], but is a novel model system for studies of CPP-membrane interactions. Binding of fluorescein-labelled peptides to PMVs was studied using both confocal microscopy and a developed protocol based on spectroscopic quantification of free and vesicle-associated peptide fractions, separated by centrifugation.

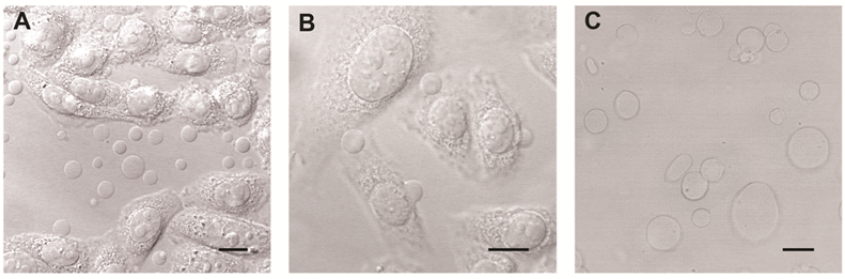


Figure 5.5 Production of plasma membrane vesicles (PMVs) from live cells.
(A), **(B)** CHO-K1 cells treated with vesiculation chemicals producing PMVs. **(C)** Purified PMVs harvested after 15 hours of vesiculation. Scale bar = 10 μm.

Figure 5.6 shows binding of the three penetratin peptides to PMVs imaged by confocal microscopy. While PenArg and penetratin show clear accumulation in the PMV membranes, PenLys displays much weaker membrane association, suggesting that the peptides indeed have different affinities for the PMV membranes. In addition, while the aim of this study was to purely monitor membrane binding (and not internalization), vesicles with more diffuse fluorescence was also observed and at longer incubations (> 30 min), membrane fragments started to appear (see Figure 2 of *Paper II*). Nevertheless, from control experiments, it was found that these vesicles were not intrinsically leaky, since low molecular weight soluble carboxyfluorescein (CF) was essentially excluded from the PMV lumen (Figure 5.7A). Furthermore, staining with an RNA-specific probe demonstrated that these vesicles contain RNA (Figure 5.7B). These findings altogether

point to that the penetratin peptides indeed have some inherent capacity to enter and also disrupt these vesicles with time, and that peptide accumulation inside PMVs could be driven by its high affinity for negatively charged RNA. It is however questionable whether the internalization observed in PMVs is occurring to high extent in live cells, where uptake is mainly endocytic (Figure 5.1).

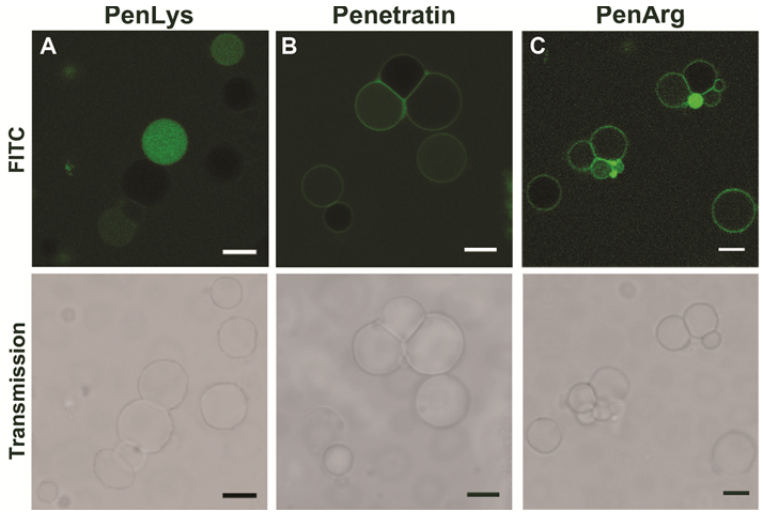


Figure 5.6 Penetratin peptides display different patterns of binding to PMVs. (A) PenLys, **(B)** penetratin, **(C)** PenArg. PenArg and penetratin show clear membrane association, while PenLys gives more diffuse fluorescence with no clear membrane localization. Images acquired 15 minutes after addition of peptide. Contrast and gain have been optimized for each image and the intensities are thus not directly comparable. Scale bar = 5 μm.

While the confocal images indicated differences in membrane affinity among the penetratin peptides, we also wanted to obtain quantitative data. This was done by briefly incubating PMVs with peptides (1-10 μM), followed by centrifugation to sediment the bound fraction, and spectroscopic quantification of the fluorescein label. As some peptide translocation was observed under the confocal microscope, control experiments were also performed to make sure that membrane binding rather than intravesicular binding (*i.e.* to RNA) was quantified. To this end, PMVs were subjected to freeze-thawing (liquid N_2/37°C) prior to peptide incubation, which will open up and reseal vesicles and hence release intravesicular content. Figure 5.8 shows the amount of peptide found in the pellet fraction as a function of total amount of peptide added to the sample, for both untreated and freeze-thawed PMVs. First of all, these are within experimental error identical, suggesting that peptide internalization does not contribute significantly to the fraction found in the pellet. Secondly, and most importantly, the penetratin peptides have strikingly different affinities for the PMVs. At both 5 and 10 μM added peptide, approximately 90% of PenArg is bound to PMVs, whereas the corresponding amount for penetratin and PenLys are ~60% and ~15% respectively, clearly showing that arginines strongly promote binding. Binding constants cannot be directly calculated from these data, since we do not know the exact nature or quantity of binding sites present, but using a simple binding equilibrium the ratio between the binding constants for PenArg and PenLys can be estimated to $K_{PenArg}/K_{PenLys} \geq 34$ (see Supplementary information of *Paper II* for derivation).

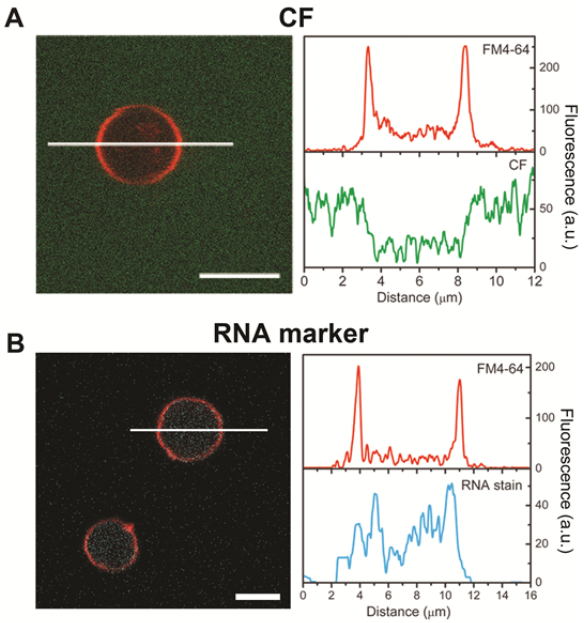


Figure 5.7 PMV membrane integrity and contents. (A) Staining with membrane marker FM4-64 (20 μg/ml) and external addition of carboxyfluorescein (CF, 5 μM). Image acquired 10 min and after addition of dyes. **(B)** Staining with FM4-64 (20 μg/ml) and RNA-specific SYTO RNASelect (3 μM) acquired after 5 min incubation. Intensity profiles along the lines indicated in the confocal images are shown in panels to the right. Scale bar = 5 μm.

The results in *Paper II* show that by using plasma membrane vesicles as a model of the cell surface, we obtain striking differences in membrane binding between the penetratin peptides. Such marked differences have not been observed when using lipid vesicles, where surface partition constants differ roughly by a factor 3 [47]. We hypothesised that an explanation for these discrepancies might be that the penetratin peptides primarily associates with proteoglycans on the cell surface. This question was indeed addressed in *Paper III* where the influence of PGs on cell surface binding and subsequent internalization of the penetratin peptides was explored (section 5.3.). However, the fact that dissociation constants for interaction with heparin *in vitro* differs only by a factor 2-3 between individual arginine and lysine residues [188] points to that cell surface interactions of CPPs involves several cell surface moieties and that the complexity and molecular organization of the cell surface therefore is important to adequately describe peptide binding.

Regardless of the molecular details involved in peptide binding to the cell surface, *Paper II* clearly demonstrates accentuated arginine-dependent differences in cell surface affinity. This means that the previous suggestions that membrane binding is not decisive for internalization efficiency of penetratin peptides needs to be re-evaluated and that cell surface binding and uptake indeed seem to correlate. The relation between cell surface binding and uptake efficiency was explored in detailed in both *Paper II* and *III* and this topic is further discussed in section 5.4.

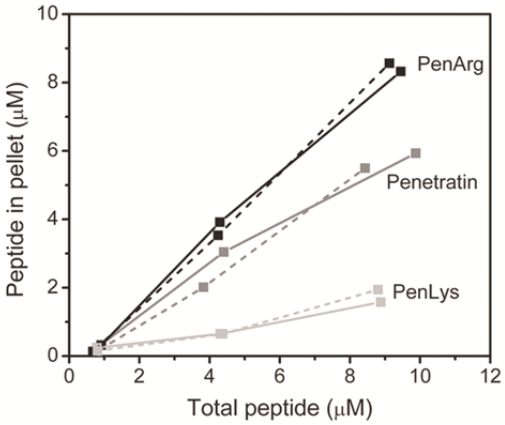


Figure 5.8 Quantification of peptide binding to PMVs. PMVs were incubated with FITC-labelled penetratin peptides for 10 min, followed by separation of bound and free peptide fractions by centrifugation. Binding curves show concentration of peptide in the pellet fraction (calculated as c_{tot}-$c_{supernatant}$) as a function of total peptide in each sample. Dashed lines represent binding to PMVs that had been subjected to 5 freeze-thaw cycles (liquid N_2/37°C) to remove intravesicular content prior to addition of peptide, while solid lines represent binding to untreated PMVs.

5.3. THE ROLE OF PROTEOGLYCANS

Proteoglycans have been suggested to be key players in the uptake of several CPPs [82-89], especially those rich in arginines [82-88], but also for penetratin [83, 88, 89]. In *Paper III*, we therefore wanted to specifically investigate the role of proteoglycans in membrane interaction and uptake of arginine- and lysine-rich penetratin versions. The dependency of penetratin uptake on these moieties was studied using wild-type CHO-K1 and proteoglycan-deficient mutant CHO-pgsA745 cells (denoted A745). The latter lacks the enzyme xylosyltransferase which is needed in the first step of glycosaminoglycan synthesis, and is hence deficient in all GAGs [189]. The ability of the penetratin peptides to bind and cluster sulfated sugars *in vitro* were also studied, using heparin as mimic for cell surface GAGs.

5.3.1. Proteoglycans in Cell Surface Binding and Uptake

The importance of cell surface proteoglycans was first assessed by comparing both cell surface association and uptake of penetratin in CHO-K1 and A745 cells. Uptake was examined in a similar manner as in *Paper I*, but instead of quantifying peptide in cell lysates, the cells were analysed by flow cytometry after treatment with heparin and trypsin, and the data represents the mean fluorescence from each counted cell. To quantify extracellular membrane binding, the protocol was adjusted so that peptide incubation was performed for 15 minutes on ice to block endocytic uptake, and in addition, cells were detached non-enzymatically to avoid peptide degradation prior to analysis. Figure 5.9 shows both cell surface binding and uptake of penetratin in concentrations ranging from 1-15 μM. What is most striking is that binding to the cell surface is virtually independent of the presence of proteoglycans, whereas uptake of penetratin is strongly enhanced when PGs are present, which is in accord with previous uptake studies in PG-deficient cells [83, 88, 89]. This

comparison was also extended to include the influence of arginines and lysines and Figure 5.10 shows binding and uptake of PenLys, penetratin and PenArg at both 5 and 10 μM concentration. Here, it is evident that arginines are beneficial for both binding and uptake, not only in wild-type CHO-K1 as observed in *Paper I* and *II*, but also in the absence of proteoglycans as shown in A745 cells. Moreover, as could be seen for penetratin in Figure 5.9, presence of proteoglycans does not significantly increase the membrane affinity of these peptides. By contrast, proteoglycans provide an enhanced capacity to internalize both lysine- and arginine-rich peptides.

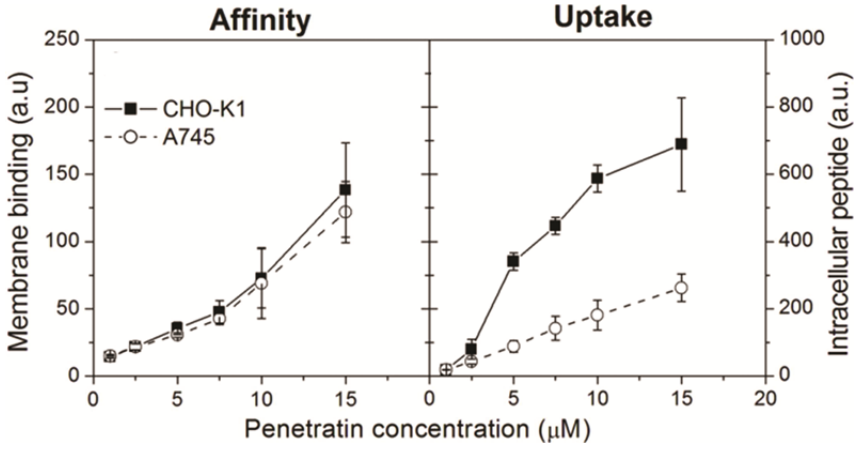


Figure 5.9 Proteoglycans increase uptake of penetratin, but not cell surface binding. Cell surface binding (left) and cellular uptake (right) of penetratin was measured as a function of peptide concentration. Cell surface binding was measured after 15 min incubation of cells at 4°C to exclude endocytic uptake. The cells were detached with non-enzymatic cell dissociation solution to avoid degradation of extracellularly bound peptide. Cellular uptake was measured after 1 h incubation at 37°C, followed by washing with heparin (100 μg/ml) to remove extracellular peptide. Experiments were performed in both CHO-K1 (filled squares) and PG-deficient A745 (open circles) cells. Binding and uptake was determined as the mean fluorescence of cells analysed by flow cytometry. The data points represent the mean (± S.D.) from 5 samples.

We further investigated whether presence or absence of cell surface proteoglycans altered the internalization pathways and hence intracellular distribution of peptide. A punctuate uptake pattern confirmed that uptake was endocytic also in the A745 cell line. However, for 10 μM PenArg, toxicity was observed, as indicated by altered cell morphology, staining with dead cell marker 7-AAD together with extensive cytoplasmic peptide localization. This toxic response was more pronounced in the A745 cells and representative confocal images are shown in Figure 5.11. Hence, our results show that PGs, in addition to promote uptake, also protect against cytotoxicity, which has previously been observed also with cationic lipids [190] and antimicrobial peptides [191].

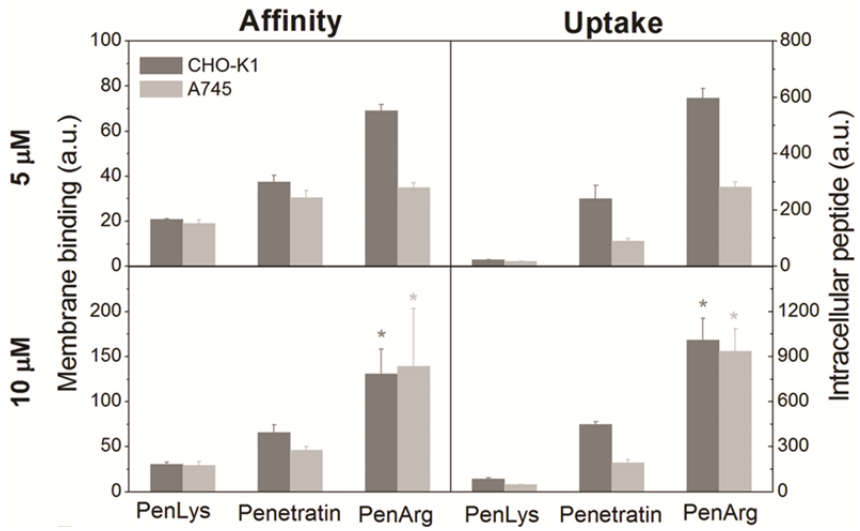


Figure 5.10 Cell uptake and binding are promoted by arginines in both CHO-K1 and A745. Cell membrane binding (left) and uptake (right) for 5 µM (top) or 10 µM (bottom) PenLys, penetratin and PenArg in CHO-K1 and PG-deficient CHO-A745 cells. Cells were incubated for 15 min at 4°C (binding) or 1 h at 37°C (uptake). The data points represent mean fluorescence intensity (± S.D.) of gated live cells from 3 separate samples. Note the different scales in the four graphs. *Peptide-mediated toxicity occurs for 10 µM PenArg (see Figure 5.11) but dead cells were excluded from the analysis.

The fact that PGs have only minor effect on the amount of membrane-associated peptide was rather surprising, since a lack of these heavily negatively charged moieties should intuitively reduce the cell surface affinity for cationic penetratin peptides. From what can be found in the literature, the only difference in cell surface composition between CHO-K1 and A745 cells is that the latter completely lack all GAG chains [189]. However, we speculated that the expression of other anionic cell membrane components could potentially be upregulated in A745 cells in order to compensate for the loss of negative PG charge and decided to address the question of overall cell surface charge experimentally by measuring the zeta potential of both CHO-K1 and A745 cells. We found a mere ~10% difference between the cell lines, which means that other negatively charged cell surface moieties such as anionic lipids or glycoproteins are indeed present at high concentrations and thereby compensate for the lack of PGs. Interestingly, PenArg was the only peptide that showed enhanced membrane binding in presence of PGs, which indicate that arginines indeed have higher affinity for PGs compared to other membrane components, but also that a high local concentration of arginines are required to promote PG binding. The latter would be consistent with previous observations that at least four arginines are required for internalization of oligoarginines [36]. However, for both penetratin and PenLys, other anionic moieties serve equally well as membrane binding sites. Therefore, the main conclusion from these experiments was that proteoglycans are primarily important for stimulating uptake of peptide at the cell surface and not for providing cell surface affinity.

A possible explanation for the uptake-triggering interactions of penetratin peptides with proteoglycans may be that they have a positive charge distribution that favour specific binding to PG chains. Heparin-binding proteins often contain consensus sequences that

result in an oriented display of positive charge upon formation of α-helix or β-sheet secondary structures [192, 193]. The penetratin peptides have indeed been shown to adopt such amphipathic secondary structures when binding to membranes [43, 104, 105] and in addition, the C-terminal domain of penetratin actually contains a heparin binding motif (N**RR**M**K**W = x**BB**x**B**x, where **B** is basic and x neutral or hydrophobic), that could potentially be involved in forming specific interactions with GAG chains on the cell surface.

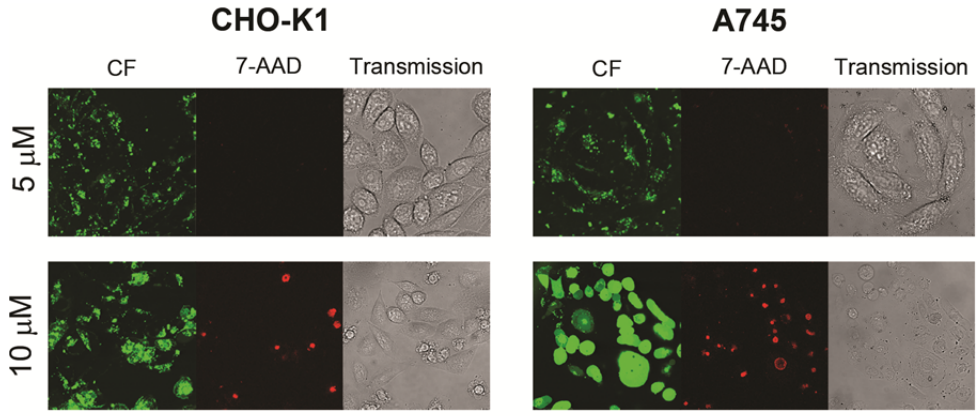


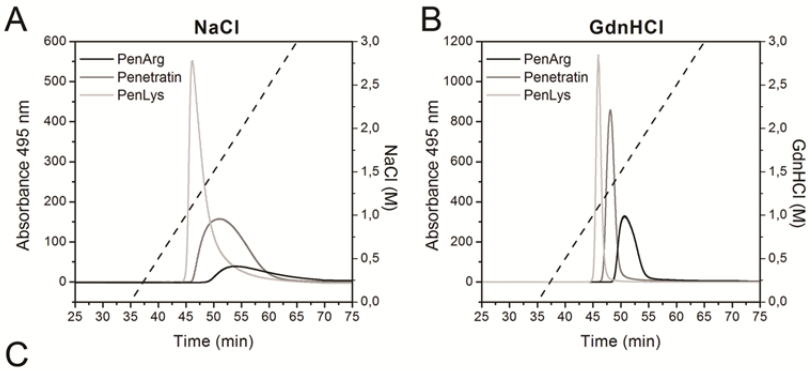
Figure 5.11 Uptake is mainly endocytic and PenArg toxicity is dependent on proteoglycans. Confocal images of live CHO-K1 (left) or A745 cells (right) incubated with 5 µM (top) or 10 µM (bottom) of PenArg for 1 h at 37°C and stained with dead cell marker 7-AAD (1 µg/ml). Contrast and gain have been optimized separately for each image. For penetratin and PenLys, a punctuate uptake pattern with no signs of toxicity was observed in both cell lines.

In conclusion, the results from *Paper III* presented above demonstrate that lysine- and arginine-rich penetratin versions can be internalized *via* both PG-*dependent* and PG-*independent* endocytosis. However, a lack of cell surface proteoglycans markedly reduce uptake, while cell surface association is mostly unaffected. These findings provide mechanistic insight regarding the role of PGs in CPP uptake and suggest that PGs, rather than having a passive role, only recruiting peptide to the cell surface, are responsible for triggering internalization of cell surface-associated peptide. In addition to this uptake-promoting role, PGs were also found to reduce peptide-mediated toxicity for PenArg, probably by preventing direct membrane interaction. Moreover, arginines were demonstrated to be beneficial for both binding and uptake, regardless of the presence of PGs. This means that arginines interact more readily also with other cell surface components, which likely contributes to their superior uptake efficiency.

5.3.2. Biophysical Studies of Peptide - Heparin Interactions

In *Paper III*, the effect of arginine and lysine substitution on the interaction between penetratin and proteoglycans was investigated also *in vitro* with heparin as a model for sulfated sugars on the cell surface. The relative heparin affinity of these peptides was assessed using heparin affinity chromatography and Figure 5.12 shows the elution profiles when either sodium chloride (NaCl) or guanidinium hydrochloride (GdnHCl) gradients (0-3 M) were used for elution. While NaCl is only capable of breaking ionic interactions,

GdnHCl is a chaotropic agent that is able to disrupt also hydrogen bonding and hydrophobic contributions to peptide-heparin binding. In addition, since guanidinium has the same chemistry as the arginine headgroup, it may be considered as a more direct competitor for arginine binding sites.



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Peptide	Eluent	Retention time (min)	Elution conc. (M)	Peak height (Abs)	Peak width w0.5 (min)*	Peak area (Abs min)
PenLys	NaCl	46	1.1	552	2.6	1889
Penetratin	NaCl	51	1.6	158	9.1	1460
PenArg	NaCl	54	1.9	39	10.6	454
PenLys	GdnHCl	46	1.1	1133	1.0	1241
Penetratin	GdnHCl	48	1.3	859	1.6	1617
PenArg	GdnHCl	51	1.6	330	3.5	1305

* The peak width at half height (w0.5) is the distance between each side of the peak measured at half the peak height.

Figure 5.12 Peptide affinity for heparin increases with arginine content. Heparin sepharose affinity chromatograms of CF-labelled penetratin peptides in 50 mM sodium phosphate buffer pH 7, eluted with 0-3 M NaCl **(A)** or GdnHCl **(B)**. The increasing salt gradient is shown as dashed lines. **(C)** Summary of the parameters retrieved from the chromatograms.

Independent of type of salt, the peptides eluted from the column in the order PenLys – penetratin – PenArg, which confirmed that arginines have higher affinity for heparin in accord with previous work comparing arginine- and lysine-rich peptides [188]. The longer retention times for arginine-containing penetratins was also accompanied by peak broadening, suggesting that PenArg and penetratin bind to heparin with a distribution of binding modes with different affinity or that binding may be cooperative. The arginine-dependent peak broadening was much more prominent with NaCl as eluent. Moreover, based on the peak area, a major fraction (~70%) of PenArg binds so strongly to heparin that it cannot be displaced at all by 3 M NaCl. Changing eluent to GdnHCl had little effect on PenLys, but resulted in shorter retention times and significantly sharper peaks for both PenArg and penetratin. PenArg was in this case completely eluted from the column. This shows that whereas PenLys interaction with heparin is strictly electrostatic, PenArg and penetratin binding is stabilized by additional contributions, such as hydrophobic interactions and hydrogen bonding. This confirms that arginines due to the guanidinium headgroup have a superior capacity to bind to sulfated sugars on the cell surface [130].

Clustering of heparan sulfate chains at the cell surface has been suggested to initiate uptake of CPPs [76, 77] and several studies have also reported CPP-induced clustering *in vitro* [78-81]. The arginine-dependent capacity of penetratin to induce clustering of heparin chains in

solution was therefore investigated by dynamic light scattering and the average hydrodynamic diameter of the formed peptide-heparin complexes are presented in Figure 5.13. Data is shown for two different charge ratios (charge ratio, *r*, equals the number of positive peptide charges divided by the number of negative heparin charges, *i.e.* $r = [+]/[–]$). Whereas all three peptides form stable complexes with an apparent diameter of ~250 nm at $r = 0.25$, both penetratin and PenArg form larger particulates, probably formed by aggregation of several complexes, at $r = 0.5$. These results are thus consistent with that arginines bind heparin with a certain degree of cooperativity and furthermore correlate qualitatively with the corresponding uptake levels of each peptide.

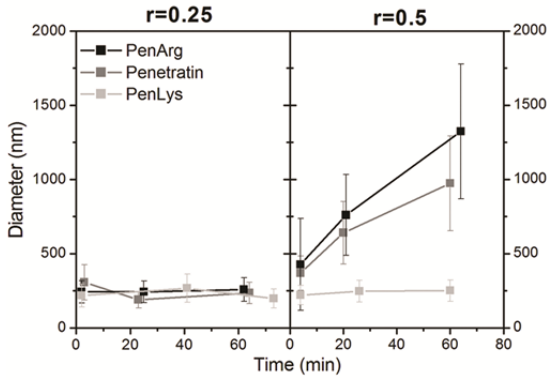

Figure 5.13 The capacity to cluster heparin is arginine-dependent. Dynamic light scattering measurements of penetratin peptides and heparin mixed at charge ratios (+/−) $r = 0.25$ (left) and $r = 0.5$ (right). A small volume of peptide was added to 10 μM heparin in 10 mM sodium phosphate/150 mM NaCl pH 7.4 and the size of the formed complexes were measured at different time-points. The data represents the mean size by intensity and the error bars the size distribution width.

In conclusion, the biophysical studies of penetratin – heparin interactions confirms that arginines bind sulfated sugars with higher affinity than lysines, and furthermore that arginines can promote clustering of several GAG chains. Both of these observations could provide a possible explanation for the superior capacity of arginine-rich peptides to bind and internalize into cells when PGs are present. However, the fact that arginines promote binding and uptake also in cells lacking PGs suggest that arginines are superior to lysines in forming favourable interactions also with other cell surface components. These questions will be discussed in the following section.

5.4. The Correlation between Binding and Uptake

The results presented in *Papers I-III* altogether suggest that arginines promote penetratin uptake both by increasing cell surface affinity and by triggering internalization. On one hand, the penetratin peptides show arginine-dependent capacities to cluster heparin *in vitro* and also to induce macropinocytosis *in vivo*, pointing towards that PenArg and PenLys differ in uptake levels due to different capacities to trigger uptake at the cell surface. On the other hand, these peptides have markedly different affinities for the cell surface, that also correlates with their uptake levels. This leads to the imperative question, which of these two factors are most important for uptake?

To address this matter, uptake was investigated as a function of cell surface binding. Such a comparison was made for the first time in *Paper II*, where uptake data from *Paper I* was plotted against peptide binding to plasma membrane vesicles (Figure 6 of *Paper II*). However, in *Paper III*, we expanded this analysis to include also the influence of proteoglycans and the results obtained from this study are shown in Figure 5.14, where uptake of 1, 5 or 10 μM of PenArg, penetratin or PenLys is plotted as a function of their corresponding cell surface binding in both CHO-K1 and A745 cells. These "uptake-binding plots" are linear (linear regressions are shown for easier comparison), showing that for each peptide the amount that is internalized corresponds directly to the amount that bound to the cell surface. Hence, there is a direct correlation between uptake and cell surface affinity. Moreover, these plots can be used to compare peptides or to compare uptake in the two cell lines. Equal slopes signify that two peptides have equal capacity to internalize into cells once bound to the cell surface. However, the fact that the magnitude of the slopes differ (see Figure 5.14) means that there are both arginine- and proteoglycan-dependent differences in the capacity to internalize membrane-associated peptides. First, the slopes are generally steeper in CHO-K1 than in A745, which means that uptake into wild-type cells is more efficient than into PG-deficient A745, even when taking into account peptide affinity for their surfaces. Furthermore, whereas the slopes are relatively similar in CHO-K1 (which is in agreement with what was found also in *Paper II* using PMVs), there are more accentuated differences between the peptides in A745 cells. Importantly, this signifies a stronger dependence on arginines for internalization of CPPs *via* non-PG binding sites.

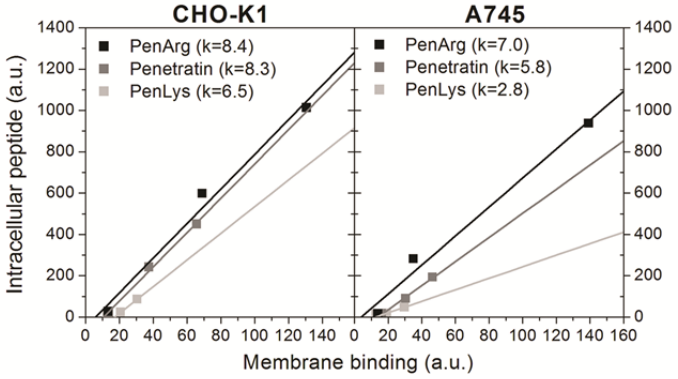

Figure 5.14 Cell uptake and binding of penetratin peptides show a linear correlation. Plotting uptake as a function of membrane binding for 1, 5 and 10 μM PenArg, penetratin or PenLys indicate the ability of each peptide to internalize into CHO-K1 (left) and A745 (right) cells once bound to the membrane. The lines represent linear regression of data and the slopes (k) are indicated in the graph legends.

In conclusion, PG-dependent uptake is primarily dependent on peptide membrane affinity, which is in accord with classical descriptions of adsorptive endocytosis [194]. Also, despite that binding cooperativity and clustering of heparin was observed for arginines *in vitro*, the linear appearance and lack of upward curvature in Figure 5.14 excludes that cooperative peptide binding events are required to trigger uptake. This further implies that the differential capacity of these peptides to trigger macropinocytosis observed in *Paper I*

primarily depends on the amount of peptide that binds to uptake-triggering sites, instead of specific interactions with arginine side chains. By contrast, uptake *via* non-PG sites depends on a combination of peptide membrane binding and the subsequent triggering of endocytic uptake, with arginines being superior for both of these events. This is important, since it means that arginines are more versatile than lysines in terms of using several portals of entry into the cell. Altogether, these results also emphasize the necessity of determining both membrane binding and uptake before drawing conclusions regarding the molecular mechanisms underlying uptake, since what might be interpreted as a specific uptake-triggering response could instead be a strong membrane binding affinity.

5.5. PENETRATIN PEPTIDES AS DNA VECTORS

While the results presented so far have focused on fundamental cell surface interactions and mechanisms behind CPP internalization, *Paper IV* instead concerns the capacity of these peptides to function as DNA delivery vectors. Non-covalent approaches for utilizing CPPs as vectors for nucleic acids are becoming increasingly popular [195], but the basic interactions between the two remains largely unexplored. In *Paper IV*, a range of biophysical techniques in combination with transfection experiments were used to investigate DNA condensation, the stability of the formed peptide-DNA-complexes ("*peptiplexes*"), and their corresponding capacity to mediate gene delivery. A systematic comparison of the seven commonly used CPPs penetratin, PenArg, PenLys, Tat, octaarginine (R8), transportan and MPG was performed. These peptides differ in net charge, arginine/lysine content and hydrophobicity (see Table 2.1 for sequences), and were chosen to address the influence of these peptide properties for non-covalent gene delivery. Since penetratin and the relative importance of arginines and lysines is the focus of this Thesis, the results for penetratin, PenArg and PenLys will be presented below, although results with the other peptides will also be referred to in order to discuss more general conclusions about the role of hydrophobic contributions and arginine content for non-covalent gene delivery.

5.5.1. Condensation of DNA

Efficient packaging of DNA into *peptiplexes* is important to facilitate uptake, but also to protect DNA from degradation by extra- and intracellular nucleases. Condensation of DNA by penetratin peptides was first assessed using flow LD to measure the gradual decrease in DNA alignment, by monitoring the LD signal at 260 nm as the DNA helix becomes condensed upon titration with peptide. Figure 5.15A shows such titrations at both low (10 mM NaCl) and physiological (150 mM NaCl) ionic strength in the regime of excess DNA (charge ratio $r = [+]/[-] < 1$). At 10 mM NaCl, all peptides show a similar condensation capacity, with a linear dependence on charge ratio and complete elimination of DNA alignment at charge ratio $r \approx 0.7$. This means that in low salt, the degree of condensation is principally dependent on electrostatic interactions between arginine/lysine side chains and DNA phosphate groups. By contrast, there is a marked difference in DNA condensing capacity at 150 mM NaCl. While PenArg is able to retain its DNA-condensing capacity, penetratin and PenLys both display less efficient DNA condensation in presence

of 150 mM NaCl. This indicates that whereas arginine interactions with DNA are not affected by the electrostatic screening of charges present at higher salt concentration, the interactions between lysine and DNA becomes significantly weakened.

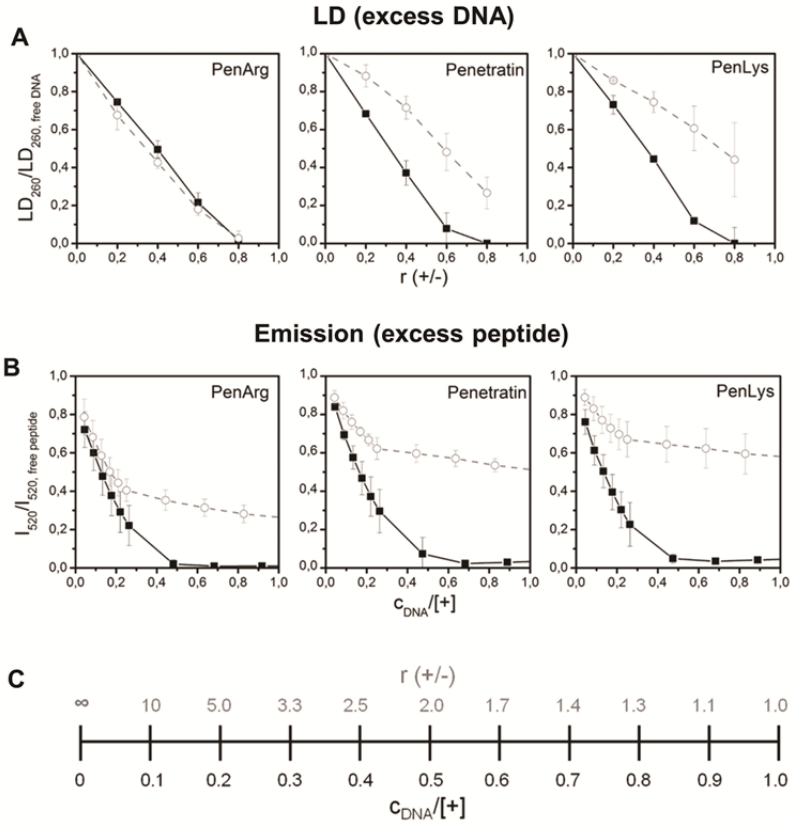

Figure 5.15 DNA condensation by penetratin peptides. (A) Condensation in the regime of excess DNA, monitored by flow LD at 10 mM NaCl (filled symbols) and 150 mM NaCl (open symbols). A small volume of peptide solution was titrated to 100 µM ct-DNA in sodium phosphate buffer (pH 7.4) containing 10 mM or 150 mM NaCl. **(B)** Binding of CF-labelled peptides to plasmid DNA in the regime of excess peptide, monitored by quenching of the CF emission at 520 nm. Small volumes of 100-300 µM plasmid DNA was titrated to 1 mL 1 µM peptide solution in sodium phosphate buffer (pH 7.4) containing 10 mM NaCl (filled symbols) or 150 mM NaCl (open symbols). **(C)** Relationship between DNA concentration and charge ratio *r*. The data in (A) and (B) are the mean of 2 or more independent experiments and error bars denote the standard deviation between measurements.

The LD experiment showed that efficient DNA condensation can be obtained already at low peptide-to-DNA ratios. However, in transfection experiments it is common to use an excess of vector to obtain positively overcharged complexes that could interact more readily with the negatively charged cell surface. In the regime of excess peptide ($r > 1$), condensation cannot be monitored by LD, since the DNA is then too condensed to align in the flow. Figure 5.15B instead shows the gradual quenching of fluorescein emission, when fluorescein-labelled peptides are titrated with increasing amounts of DNA. Quenching of the fluorescein peptide label upon complex formation with DNA may be explained by self-quenching of proximate fluorescein molecules [196] and we found support for this view by observing that quenching was accompanied by absorption broadening, indicative of fluorophore-fluorophore interactions. We also verified that the

degree of quenching was in qualitative agreement with the extent of peptide binding, by separating bound and free peptide fractions by ultracentrifugation. The quenching experiments were performed at both 10 mM NaCl and 150 mM NaCl, in analogy with the LD experiments. However, since these titrations were done with increasing DNA-to-peptide charge ratio, the degree of quenching is plotted as a function of inverse charge ratio r (*i.e.* $1/r = c_{DNA}/[+]$). For easier comparison between LD and quenching data, Figure 5.15C shows how the inverse charge ratio converts to charge ratio. In agreement with the LD experiments, the peptides behave similarly at 10 mM NaCl, with quenching increasing almost linearly with inverse charge ratio up to $1/r \approx 0.5$, where the emission becomes completely quenched. At 150 mM NaCl, we again observe an arginine-dependent capacity to interact with DNA. The degree of quenching is in the order PenArg > penetratin > PenLys. In addition, the total quenching is considerably less efficient and the curves level off before reaching zero, indicating weaker interactions for all three peptides. This suggests that significant amounts of free peptide will exist at the point of charge neutralization ($r = 1$). This was not observed in LD, where the total concentrations of peptide and DNA were hundredfold higher, thereby shifting the equilibrium towards formation of peptiplex.

Taken together, by studying DNA condensation with two complementary techniques, we show that arginines form intrinsically stronger bonds with DNA resulting in more efficient peptiplex formation. This can be attributed to that the guanidinium headgroup of arginine side chains form bidentate hydrogen bonds with phosphates in the DNA backbone, whereas the lysine amine group binds *via* ionic attraction.

5.5.2. Stability of Peptide-DNA Complexes

While the ability to form complexes with DNA may be considered as a first crucial step towards non-covalent DNA delivery, the next challenge is for these complexes to remain intact and protect DNA in a cellular milieu. The stability of the formed peptiplexes was assessed *in vitro* using two different model systems to mimic cellular environments. First, the putative stability of peptiplexes in presence of cellular surfaces was investigated by subjecting peptiplexes to heparin. Second, the capacity of the peptides to protect DNA from enzymatic degradation was examined.

Peptiplex stability in presence of heparin was investigated using flow LD to monitor decompaction and re-alignment of released DNA. Figure 5.16A shows the change in DNA orientation as a function of time after addition of heparin to peptiplexes formed at $r = 0.6$, yielding equimolar charge concentrations of heparin and DNA. Figure 5.16B shows the initial (white bars) and final (grey bars) levels of aligned DNA from the kinetic traces. At 10 mM NaCl, the penetratin peptides all condensed ~75-80% of the available DNA, but showed an arginine-dependent behaviour in heparin-induced dissociation with PenArg releasing about 60% of the complexed DNA fraction and PenLys showing almost complete release. At 150 mM NaCl there is a substantial difference in initial degree of DNA compaction, in accord with Figure 5.15, which also means that the starting values before dissociation are different. PenArg is again least affected by salt, with equal level of

compaction and an even lower level of DNA release (~35% of complexed fraction) than at 10 mM NaCl. Penetratin and PenLys showed markedly different initial compaction, but both released virtually all DNA upon exposure to heparin. This suggests that penetratin and PenLys have a much higher relative affinity for heparin compared to DNA and may therefore dissociate from the DNA upon encounter with the cell surface. By contrast, a substantial fraction of peptiplexes formed with PenArg is resistant to heparin and may therefore have a higher probability of remaining intact upon encountering the cell surface. In summary, this means that PenLys is insufficient as a DNA delivery vector whereas penetratin shows satisfactory DNA condensation but limited stability, and PenArg is able to efficiently condense DNA into complexes that also appear sufficiently stable.

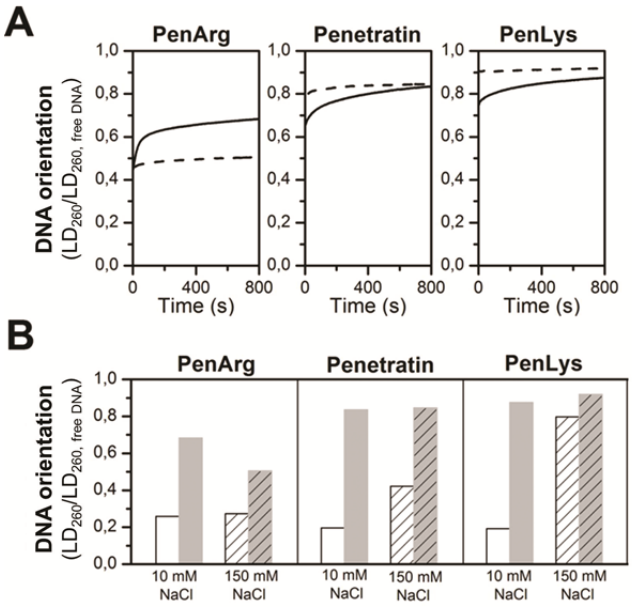

Figure 5.16 Stability of peptiplexes in presence of heparin (A) Heparin-induced dissociation of peptides from ct-DNA at 10 mM NaCl (solid line) and 150 mM NaCl (dotted line), monitored by flow linear dichroism. Peptide was added to ct-DNA (100 µM) at a charge ratio r = 0.6 and incubated for 10 minutes before exposure to heparin (100 µM negative charges). The LD signal for DNA (at 260 nm) was recorded before and after condensation with peptide, and then constantly monitored for at least 20 minutes following the exposure to heparin. **(B)** Extent of DNA condensation obtained with each peptide before and after exposure to heparin, at 10 mM NaCl (plain bars) and at 150 mM NaCl (striped bars). The white bars denote the amount of DNA orientation remaining after condensation with peptide (*i.e.* the starting value in the kinetic curves) and grey bars denote the final amount of DNA orientation in the sample (*i.e.* the final value in the kinetic curves). Data is representative of two or more independent experiments.

The ability of the peptides to protect DNA from enzymatic degradation was assessed by exposing peptiplexes to serum for 3 h, followed by inactivation of serum nucleases, dissociation of complexes with heparin/SDS and analysis by gel electrophoresis. If the peptiplexes are sufficiently dense, nucleases will be sterically excluded and the DNA will remain intact. Figure 5.17 shows representatives gels and quantification of DNA bands, when DNA had been complexed with penetratin peptides at charge ratios from r = 1 to r = 10. Whereas uncomplexed DNA was completely degraded under these conditions, the penetratin peptides all provided substantial protection with typically 20-30% of the DNA remaining fully intact at r = 2 and around 50% at r = 10. In contrast to the heparin

dissociation experiments, there are no apparent differences in serum stability between these peptides. This suggests that arginines and lysines form complexes of comparable density and more generally that high affinity for DNA does not necessarily result in more efficient protection.

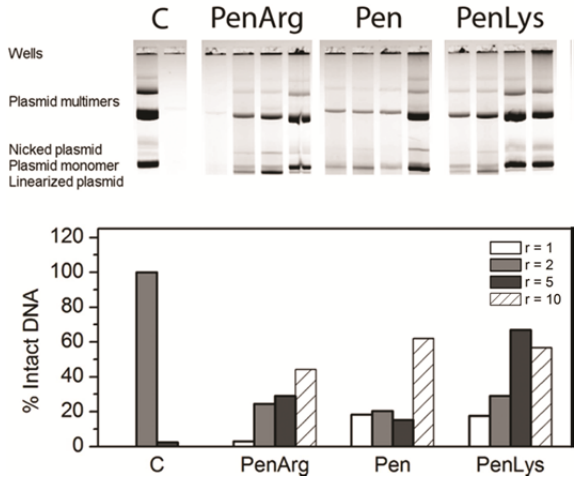

Figure 5.17 Resistance of peptiplexes towards enzymatic degradation. The stability in presence of serum was assessed by gel electrophoresis after dissociation of complexes. Lanes represent intact DNA remaining in the samples after condensation with peptides at charge ratios r = 1, 2, 5, and 10 from left to right. C: Control samples with intact DNA and with naked DNA exposed to serum nucleases, respectively. Data is representative of three or more independent experiments.

The data presented above demonstrate that arginines are superior to lysines in condensing DNA into complexes that are stable towards dissociation. However, comparison with the two arginine-rich CPPs Tat and R8 (see Table 2.1) that were also investigated in *Paper IV*, clearly shows that a high arginine content is not sufficient to promote DNA binding. Instead, both Tat and R8 were found incapable of binding DNA at physiological salt concentration in dilute systems, and also dissociated completely from DNA upon competition with heparin (see Figure 4 and 6 of *Paper IV*). By contrast, the most hydrophobic peptide studied in *Paper IV*, transportan (Table 2.1), is also most resistant towards heparin, meaning that dissociation in presence of heparin is less efficient when peptiplexes are stabilized by hydrophobic interactions (Figure 6 of *Paper IV*). This suggests that in addition to arginines, hydrophobic residues are also required for strong DNA binding. PenArg may be a good compromise in this respect, since it in addition to a high arginine content also is moderately hydrophobic. Another important difference between PenArg and R8/Tat is the spacing of positive charges. This could be important for favourable DNA interactions, something that has indeed been observed for polyamidoamines [197]. Further support for the importance of hydrophobic residues is provided by the fact that the two most hydrophobic CPPs assessed in *Paper IV* (transportan and MPG, see Table 2.1) were superior in terms of DNA protection against enzymatic degradation, which clearly demonstrates the need for hydrophobic interactions to obtain sufficiently dense complexes, whereas arginine content in this case seems insignificant (see Figure 7 of *Paper IV*).

5.5.3. Peptide-Mediated Gene Delivery

The biophysical data presented above collectively give an indication of the potential of these peptides to package DNA so that it can be intactly delivered into cells. Their true capacity to do so was also assessed in *Paper IV* by exposing HEK293T cells to peptiplexes prepared by mixing pEGFP-C1 plasmid DNA, encoding enhanced green fluorescent protein (eGFP, [179]), with each peptide at charge ratios $r = 5$ and $r = 10$, followed by flow cytometry analysis to quantify eGFP-expressing cells 24 h later. Transfection was performed in presence of endosome-disruptive chloroquine, which eliminates differences in transfection efficiency due to differential capacities to escape from endosomes. This simplifies comparison of the CPP capacity to mediate uptake of DNA, but rules out the influence of events downstream internalization. Figure 5.18 shows the result from these experiments, comparing the transfection efficiency of peptiplexes with that of naked DNA and the commercially available cationic lipid reagent Lipofectamine™. Whereas PenArg transfects ~25-30% of the cells at both charge ratios, penetratin transfects ~20% at $r = 10$, but is not significantly better than naked DNA at $r = 5$, and PenLys is essentially non-functional at both charge ratios. The ability to mediate transfection is thus arginine-dependent and furthermore in qualitative agreement with each peptide's intrinsic uptake propensity as well as its ability to form stable, heparin-resistant complexes with DNA.

The need for a combination of both efficient uptake and favourable peptide-cargo interactions was further confirmed by comparison with other CPPs investigated in *Paper IV*. The arginine-rich R8 and Tat were, despite being associated with efficient internalization [36, 55], virtually non-functional as non-covalent DNA vectors, probably due to inability to confer peptiplex stability. By contrast, the hydrophobic peptides transportan and MPG, which like PenArg combine efficient internalization [30, 33] with peptiplex stability, also show high transfection efficiency (see Figure 2 of *Paper IV*). This again supports that arginines are better than lysines, but in the case of DNA condensation and delivery, hydrophobic interactions are critical. Indeed, this has been realized also by others and chemical modifications aimed to increase hydrophobicity such as stearylation, have resulted in enhanced nucleic acid delivery of arginine-rich peptides [122-124]. However, our results suggest that a more flexible and versatile approach to enhance transfection efficiency of arginine-rich CPPs would be to incorporate hydrophobic amino acid residues. Alternatively, one could substitute lysines for arginines in hydrophobic peptides such as transportan and MPG. The work presented in this Thesis certainly show that the latter approach has been successful for penetratin, and a few other studies have also shown improved uptake or transfection with CPPs that have been modified to incorporate more arginines [198-201] or guanidinium groups [202].

In conclusion, *Paper IV* clearly demonstrates that arginines are superior to lysines in condensing DNA into complexes that are stable towards dissociation in presence of sulfated sugars, and this capacity correlates with the ability to mediate uptake and expression of plasmid DNA *in vitro*. In addition to arginines, we also highlight hydrophobic residues as key components in stabilizing CPP-DNA interactions. Our results provide

mechanistic insights into how different physico-chemical peptide properties contribute to successful transfection, with important implications for CPP design.

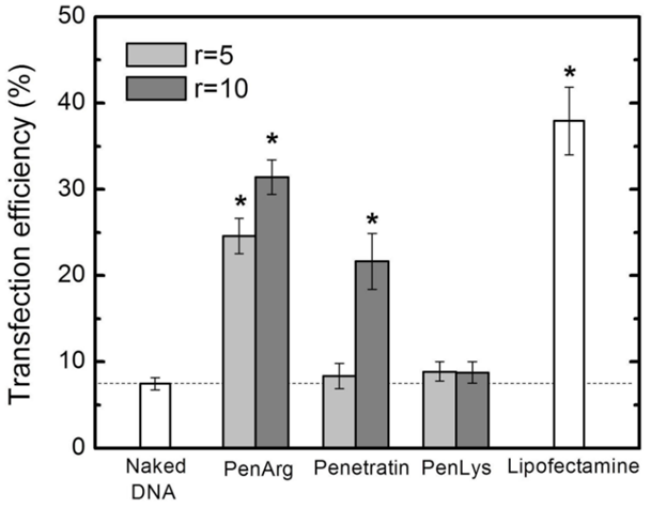

Figure 5.18 Transfection efficiency of penetratin peptides in HEK293T cells. Plasmid pEGFP-C1 was condensed with peptides at charge ratios 5 (light grey) and 10 (dark grey), and added to cells in presence of chloroquine. The number of eGFP-expressing cells was assessed by flow cytometry 24 hours after transfection. Dead cells were excluded from the analysis via 7-AAD staining. Column height represents the average of four independent experiments performed in triplicate (N=4, n=3), and error bars represent the SEM. Transfection with negative control (naked DNA) and positive control (Lipofectamine™) are shown for reference. Statistically significant difference from naked DNA was assessed by a paired Student's t test; * $p < 0.01$.

5.5.4. Improving Gene Delivery with Cysteine

A potential strategy to improve non-covalent CPP-mediated gene delivery is to enhance complex stability by forming peptide multimers. In order to obtain reversible DNA condensation, an approach based on addition of cysteine residues has been explored for several peptides, including lysine-rich [203] or arginine- rich [204] peptides, Tat [205] and the endosomolytic KALA peptide [206]. In addition, transfection with MPG has been shown to increase 1000-fold upon addition of a C-terminal cysteamide [61]. This strategy enables reversible generation of peptide multimers by formation of disulfide bridges that can subsequently be reduced in the intracellular environment and thereby aid DNA release [207]. In addition, this approach provides advantages in terms of peptide production, since large peptides are expensive and hard to synthesise, and repeated sequences are often difficult to express recombinantly [208].

With the aim to improve penetratin for non-covalent gene delivery, we decided to explore the strategy of cysteine addition. To this end, a number of penetratin-based analogs were designed by adding a C-terminal cysteine, and efficient, reversible formation of dimers was verified by gel electrophoresis (SDS-PAGE), performed under both reducing and non-reducing conditions (Figure 5.19). Cysteine addition was first assessed for penetratin itself and in the presence of endosome-disruptive chloroquine, a C-terminal cysteine improved transfection by approximately a factor 2 (Figure 5.20A). The same experiments were repeated also in the absence of chloroquine, but cysteine modification did not improve

endosomal escape (Figure 5.20B). Addition of cysteine in the N-terminus or in both ends (resulting in multimer formation) did not influence transfection efficiency compared to C-terminal modification of penetratin (data not shown).

As a next step, a C-terminal cysteine was added also to PenArg, which had previously been shown to be superior compared to penetratin in mediating gene delivery (Figure 5.18). In presence of chloroquine, cysteine addition dramatically enhances transfection at $r = 2$ by approximately a factor 6 compared to PenArg (Figure 5.20A). However, with peptide in large excess ($r = 10$), PenArg and PenArg-Cys instead show equal transfection efficiencies, indicating that PenArg at this charge ratio is able to form stable peptiplexes even without cysteines. As for penetratin, cysteine addition to PenArg did not enhance endosomal escape (Figure 5.20B).

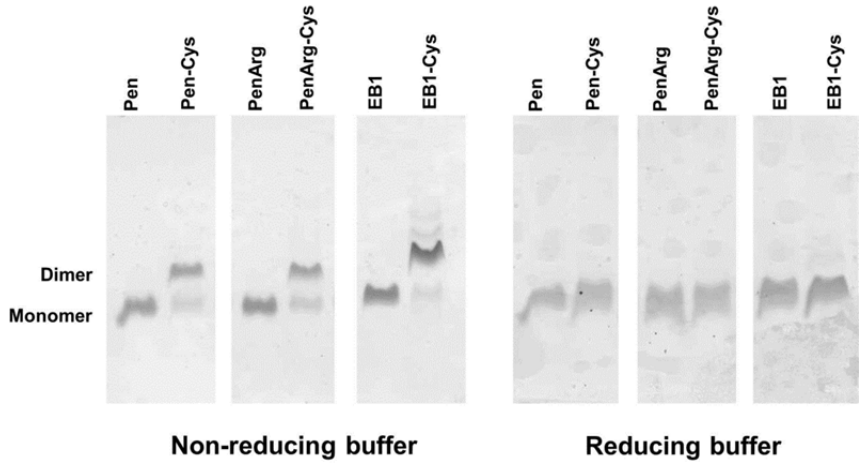

Figure 5.19 SDS-PAGE of CPPs. Addition of a C-terminal cysteine results efficient and reversible dimer formation in solution.

Lack of endosomal escape is naturally a severe limitation for delivery of cargo into the cell. Therefore, the next step towards optimizing penetratin for gene delivery was to explore the penetratin analog EB1 (see Table 2.1), which is N-terminally extended and substituted with basic histidines (pK_a ~6), in order to yield an amphipathic α-helix upon protonation in the acidic endosomal environment. EB1 has previously been shown to be superior compared to penetratin in forming complexes and deliver biologically active siRNA into cells [39], but has not been used for transport of plasmid DNA. In presence of chloroquine, EB1 was able to transfect ~40-50% of the cells, even at a charge ratio of 2 (Figure 5.20A). Compared to penetratin and PenArg, EB1 thus shows markedly better transfection capacity independent of charge ratio. Addition of a C-terminal cysteine to form EB1-Cys, did not improve transfection efficiency further, indicating that with EB1, sufficiently stable peptiplexes are formed without cysteines. However, the most striking result was obtained in the absence of chloroquine, where the supposedly endosomolytic EB1 peptide was unable to mediate transfection, but EB1-Cys transfected a substantial amount of cells (Figure 5.20B). By contrast, without chloroquine, the transfection efficiency of EB1-Cys was strongly dependent on charge ratio, with less than 5% transfection at $r = 2$, and ~10% at $r = 10$.

In summary, cysteine modification has different effects depending on peptide sequence, charge ratio and presence of chloroquine. In the absence of chloroquine, cysteine modification improves transfection with penetratin and PenArg at low charge ratios. This could be explained by stabilization and enhanced uptake of peptiplexes, but in addition, chloroquine has, besides buffering the pH of endocytic vesicles, also been found to induce dissociation of vector-DNA complexes [209, 210]. This means that chloroquine could have a dual role in enhancing transfection, by improving both endosomal escape and DNA unpacking. The fact that PenArg-Cys, EB1 and EB1-Cys all show highest transfection at $r = 2$, with a slight decrease at $r = 10$, suggests that these peptides form stable complexes already at low charge ratios and that a large excess of peptide instead inhibits DNA release. In the absence of chloroquine, EB1, which was designed to be endosomolytic, was essentially non-functional, while EB1-Cys showed markedly enhanced transfection capacity. This points to that whereas EB1 is unable to exert its endosomolytic function when bound to plasmid DNA, EB1-Cys more readily interact with the endosome membrane and induce endodomal escape. This could be due to an enhanced membrane affinity of EB1-Cys dimers, compared to EB1 monomers. Alternative explanations could be that EB1 in absence of chloroquine have reduced capacity to dissociate from DNA, or that cysteine addition indeed enhances stability and uptake of peptiplexes, but that this was not detectable when endosomal escape was induced, due to the large amount of peptiplexes that was released into the cytosol.

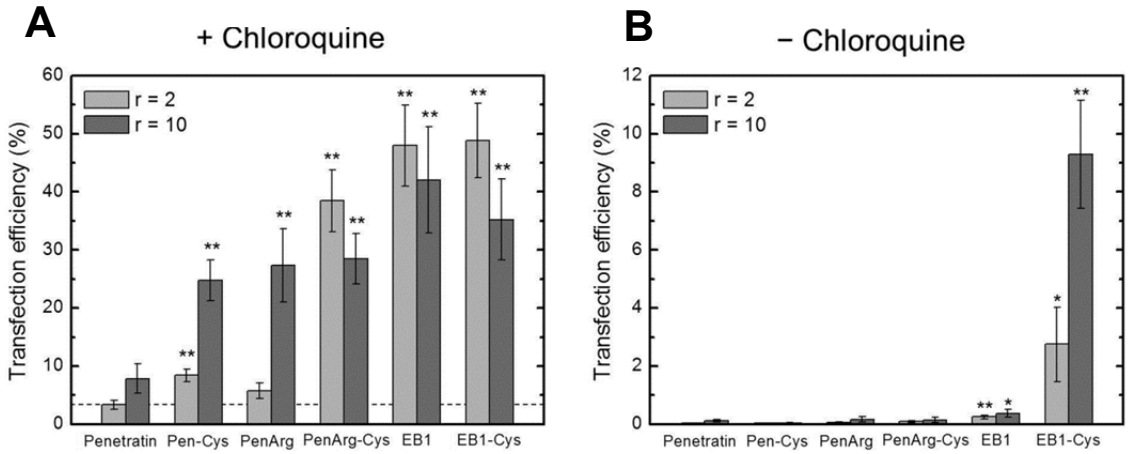

Figure 5.20 Transfection efficiency in HEK293T cells. Plasmid pEGFP-C1 was condensed with peptides at charge ratio 2 (light grey) and 5 (dark grey) and added to cells in presence **(A)** or absence **(B)** of chloroquine. The percentage of cells expressing the transgene eGFP was assessed 24 h after transfection. Dead cells were excluded from the analysis via 7-AAD staining. Peptiplexes were prepared with 0.2 μg DNA per well. The presented data is the mean (± SEM) of at least 3 independent experiments performed in triplicate. Positive control (Lipofectamine™) yielded 22±8% in presence and 19±4% in absence of chloroquine. Statistically significant difference from naked DNA (dashed line) was assessed by paired Student's T-test, $p < 0.05$ (*) and $p < 0.01$ (**).

The promising transfection results obtained upon cysteine addition prompted us to further investigate the potential of these peptides for gene delivery. Another important aspect is that the vector is non-toxic to cells and this was assessed using the LDH leakage assay. This assay is based on measurement of lactate dehydrogenase (LDH) activity in the

extracellular medium as LDH leaks out in response to membrane damage [211]. Regardless of chloroquine addition, both penetratin and PenArg peptiplexes were virtually non-toxic to cells and cysteine addition only shows a slight tendency to enhance PenArg cytotoxicity (Figure 5.21). By contrast, EB1 peptiplexes show substantial concentration-dependent cytotoxicity, with between ~10-40% leakage upon incubation for 2 h. Importantly, EB1-Cys peptiplexes show considerably lower toxicity than the parent peptide EB1, with approximately half the amount of leakage. The lower cytotoxic response with EB1-Cys could be due to a higher affinity and hence tighter binding to DNA, which would reduce the amount of free peptide available for direct interaction with the plasma membrane. In summary, this means that by addition of a single cysteine residue to EB1, we have gone from a relatively toxic peptide that is trapped inside endosomes, to a peptide that can perform endosomal escape and mediate gene delivery in the absence of endodome-disruptive chemicals, without causing cytotoxic side-effects.

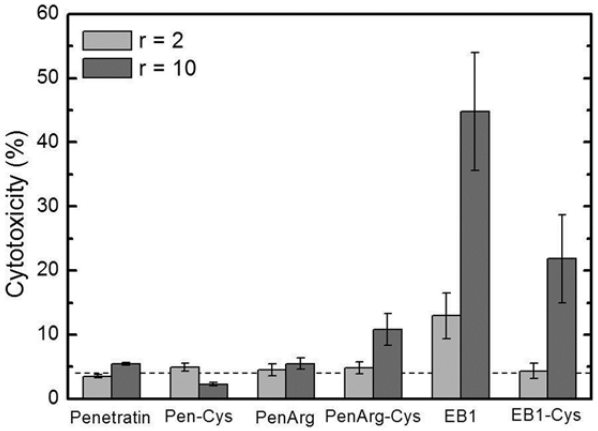

Figure 5.21 Cytotoxicity of peptiplexes. Plasmid pEGFP-C1 was condensed with peptides at charge ratio 2 (light grey) and 5 (dark grey) and added to cells. Cytotoxicity was measured by LDH release after 2 h exposure to peptiplexes. The presented data is the mean (± SEM) of at least 3 independent experiments performed in triplicate. Dashed line represent LDH leakage from negative control (no peptide added).

In conclusion, the results from *Paper V* demonstrate that addition of a single cysteine residue to enable peptide dimer formation have the capacity to markedly improve stability, enhance endosomal escape and reduce cytotoxicity of peptiplexes. These factors are all critical for succesful gene delivery and cysteine modification is therefore a promising strategy to improve CPPs as non-covalent delivery vectors.

6. CONCLUDING REMARKS

Cell-penetrating peptides are certainly promising as intracellular delivery vectors due to their well-established capacity to enter cells and deliver macromolecular cargos in a non-toxic manner. Still, there are many challenges on the way towards successful clinical application and these include enhancing uptake efficiency, improving endosomal escape and targeting specific tissues or cells. In addition, the complex nature of biopharmaceutical cargos will most likely require individualized optimization of delivery systems. CPPs are adaptable in this way, since the amino acid sequence can easily be modified to obtain new, improved peptide functionalities. An alternative approach that might prove most successful is to combine CPPs with other delivery agents to obtain multi-component systems that combine packaging of cargo, cell targeting, uptake, and endosomal escape. Today, several CPP constructs have reached clinical trials, but despite this, we are still striving to understand fundamental concepts regarding their uptake and delivery of cargo into cells. Therefore, mechanistic *in vitro* studies that aim at understanding and improving CPP function need to be performed in parallel with *in vivo* studies that continue to evaluate their therapeutic potential. Such a dual approach will hopefully make way for CPPs in future therapeutic applications.

This Thesis deals with fundamental concepts regarding the mechanism by which CPPs function. Such mechanistic insight is, in addition to being central for development of CPPs, also interesting in a broader scientific context, since interactions with and uptake over the plasma membrane are fundamental biological processes. The work presented here addresses three key steps in the mechanism of CPPs: *membrane binding*, *stimulation of uptake* and *gene delivery,* using the classical CPP penetratin as a model peptide. In addition, the molecular prerequisites for CPP function are studied, by specifically investigating the importance of arginines and lysines in the peptide sequence, and proteoglycans (PGs) on the cell surface. A general observation throughout the work of this Thesis is that arginines possess characteristics that make them superior to lysines in almost all aspects that are important for CPP function.

Papers I-III explore uptake of penetratin peptides from a mechanistic perspective, by comparing uptake efficiency with the corresponding capacity to (1) bind to the cell surface and (2) stimulate macropinocytosis. First, in *Paper I* we show quantitatively that PenArg has a greater capacity than PenLys to trigger macropinocytic internalization. In *Papers II-III* we search for explanations for this greater uptake-triggering capacity by quantifying peptide binding to the cell surface and find that cell surface affinity is also strongly arginine-dependent. Moreover, we demonstrate that the amount of peptide that resides at the cell

surface is the primary factor that determines penetratin uptake efficiency. This means that the greater capacity of arginines to trigger macropinocytosis and internalize into cells first and foremost have a pure physico-chemical explanation where the determining factor for uptake is the amount of peptide that binds to uptake-triggering sites, rather than specific interaction with arginine side chains. Importantly, this means that arginines and lysines can promote uptake by the same mechanisms, although arginines more efficiently, due to a higher capacity to bind to the cell surface.

In *Paper II*, we introduce plasma membrane vesicles (PMVs) as a novel membrane model in CPP research. The most important conclusion from this study is that by using a model system that is similar to the plasma membrane in both composition and organization, we are able to find a direct correlation between membrane interaction and uptake capacity of CPPs. This is important, since such correlations have been difficult to find using less complex models for the cell surface including lipid membranes or soluble sulfated sugars. This clearly demonstrates that cell surface interactions of CPPs are dependent on several cell surface groups and their molecular organization at the membrane surface. In addition to contributing to our understanding of CPP uptake, this finding will hopefully lead the way in development of more cell-like model systems in studies of membrane interactions.

The role of proteoglycans in uptake of CPPs has been a subject of intense investigation during the last decade and this topic was addressed in *Paper III*. The results from this work show that PGs are important for uptake of penetratin peptides, in accord with previous observations in the literature. However, since lack of proteoglycans has only a minor effect on cell surface binding, the role of PGs in CPP uptake is primarily to trigger internalization, not to recruit peptide to the cell surface. By contrast, we demonstrate that the capacity to internalize cell surface-associated peptide in absence of proteoglycans is arginine-dependent, meaning that arginines are less reliant on PGs for internalization and hence more versatile than lysines in terms of promoting uptake *via* multiple pathways. In conclusion, the superior uptake efficiency of arginine-rich CPPs can thus be traced back to a combination of high cell surface affinity and uptake-triggering capacity. However, we also identify one potential drawback with arginine-rich CPPs, and that is the risk of cytotoxicity at elevated peptide concentrations. Importantly though, we also find that cell surface proteoglycans can reduce this toxic response, which means that these moieties have a dual role in CPP-mediated uptake in both promoting internalization and protecting against invasive membrane interactions.

Papers IV-V focus on interactions between CPPs and DNA and the capacity to mediate gene delivery using a non-covalent approach. In *Paper IV*, we explore gene delivery from a mechanistic perspective by investigating how the physico-chemical properties of CPPs influence their ability to condense DNA into stable peptiplexes that can mediate cell transfection *in vitro*. We demonstrate that arginines are superior to lysines also in this respect, but in addition, we find that hydrophobic contributions are needed to stabilize peptide-DNA interactions, and are thus decisive for successful gene delivery. Based on these results, we suggest substitution of lysines for arginines or introduction of hydrophobic residues in existing CPP sequences as a strategy to create novel efficient

vectors. The latter would be a more versatile approach than conjugation with hydrophobic stearyl or cholesterol derivatives, which has previously been explored for arginine-rich CPPs. In *Paper V*, we investigate an additional strategy to enhance gene delivery, and that is incorporation of cysteines into CPPs. We show that addition of a single cysteine residue can improve stability, enhance endosomal escape capacity, and reduce cytotoxicity of the formed peptiplexes.

In conclusion, this Thesis identifies several key properties for successful gene delivery with CPPs and that is a high *arginine* content to enhance DNA condensation and promote internalization, *hydrophobic* residues to stabilize peptide-DNA interactions, and a terminal *cysteine* to further improve peptiplex stability and enhance endosomal escape. These mechanistic insights provide important implications for design of peptide-based gene delivery systems in the future.

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I år är det precis 50 år sedan Nobelpriset i medicin tillägnades upptäckten av DNA:s struktur, som ligger till grund för förståelsen av vår arvsmassa. Sedan dess har utvecklingen inom molekylärbiologi och genetik gått i ett rasande tempo, och idag har vi möjlighet att identifiera *gener* som orsakar olika sjukdomar. Denna kunskap gör det också möjligt att behandla sjukdomar på ett helt nytt sätt, genom att föra in nya, friska gener i våra celler, vilket kallas *genterapi*.

Ett av de största hindren för utvecklingen av genterapeutiska behandlingsmetoder är att DNA måste ta sig över cellens skyddsbarriär; cellmembranet. För att DNA, som är en stor och negativt laddad molekyl, ska kunna ta sig över membranet på ett effektivt sätt behövs en *vektor* – en slags bärarmolekyl. Denna avhandling handlar om en sorts molekyler som skulle kunna fungera just som vektorer för DNA: *Cell-penetrerande peptider*. Dessa är korta aminosyrasekvenser (d.v.s. små proteiner) som har visat sig ha den sällsynta förmågan att ta sig över membranbarriären och in i levande celler.

Forskningen som presenteras i denna avhandling syftar till att förstå *hur* cell-penetrerande peptider fungerar genom att studera hur den kemiska strukturen hos peptiden påverkar dess förmåga att ta sig in i cellerna och leverera DNA. Jag visar att genom att byta ut en aminosyra (lysin) mot en annan (arginin), kan en peptid öka sin förmåga att binda till cellmembranet och stimulera upptag genom endocytos – en av cellens naturliga mekanismer för att ta upp molekyler. Dessutom visar jag att peptiden med högre upptagseffektivitet också är bättre på att binda till och mediera transport av DNA in i celler. Min forskning bidrar därmed till värdefulla insikter kring hur cell-penetrerande peptider ska kunna utvecklas och designas för att bli effektiva vektorer för genterapi.