When cationic cell-penetrating peptides meet hydrocarbons to enhance in-cell cargo delivery

Margherita Di Pisa, Gérard Chassaing, Jean-Marie Swiecicki

When cationic cell-penetrating peptides meet hydrocarbons to enhance *in cell* cargo delivery

Margherita Di Pisa[a],[b],[c]*, Gérard Chassaing[a],[b],[c] and Jean-Marie Swiecicki[a],[b],[c]*

[a] Sorbonne Universités, UPMC Univ Paris 06, UMR 7203, Laboratoire des Biomolécules, F-75005, Paris, France

[b] CNRS, UMR 7203, Laboratoire des Biomolécules, F-75005, Paris, France

[c] ENS, UMR 7203, Laboratoire des Biomolécules, Département de Chimie, Ecole Normale Supérieure, 24 Rue Lhomond, F-75005, Paris, France

E-mail: jean-marie.swiecicki@ens.fr, margherita.dipisa@ens.fr

Author contributions:

* These authors contributed equally and are corresponding authors: Margherita Di Pisa and Jean-Marie Swiecicki

Abstract

Cell penetrating peptides (CPPs) are short sequences often rich in cationic residues with the remarkable ability to cross cell membranes. In the past 20 years, CPPs have gained wide interest and have found numerous applications in the delivery of bioactive cargoes to the cytosol and even the nucleus of living cells. The covalent or non-covalent addition of hydrocarbon moieties to cationic CPPs alters the hydrophobicity/hydrophilicity balance in their sequence. Such perturbation dramatically influences their interaction with the cell membrane, might induce self-assembling properties and modifies their intracellular trafficking. In particular, the introduction of lipophilic moieties changes the subcellular distribution of CPPs and might result in a dramatically increase of the internalization yield of the co-transported cargoes. Herein, we offer an overview of different aspects of the recent findings concerning the properties of CPPs covalently or non-covalently associated to hydrocarbons. We will focus on the impact of the hydrocarbon moieties on the delivery of various cargoes, either covalently or non-covalently bound to the modified CPPs. We will also provide some key elements to rationalize the influence of the hydrocarbons moieties on the cellular uptake. Furthermore, the recent *in vitro* and *in vivo* successful applications of acylated CPPs will be

summarized to provide a broad view of the versatility of these modified CPPs as small-molecules and oligonucleotides vectors.

Keywords

cell penetrating peptide, lipopeptide, acylated peptide, vesicle, drug delivery, transfection, targeted drug delivery, pyrenebutyrate

List of abbreviations

12-Aminododecanoic acid (Ado); 6-Aminohexanoic acid (Ahx); 5-Aminopentanoic acid (Ava); Blood-Brain Barrier (BBB); Cell Penetrating Peptides (CPPs); Critical Micelle Concentration (CMC); 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC); 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE); 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoserine (DOPS); Green Fluorescent Protein (GFP); Linear Peptides Amphiphiles (LPAs); Multifunctional Envelope-Type Nano Device (MEND); Phosphate-Buffered Saline (PBS); Phospholipids (PLs); Phosphoramidate Morpholino Oligonucleotides (PMO); Polyethylene Glycol (PEG); Pro-Apoptotic Domain (PAD); Pyrenebutyrate (PyB); Red Fluorescent Protein (RFP); Small interfering RNA (siRNA); Sphingomyelin (SM); Solid Phase Peptide Synthesis (SPPS)

Introduction

Small molecule-based drugs have been very successful over the past 60 years in interfering with biological processes. Despite the considerable size of the chemical space and the always growing-up *savoir-faire* of chemists, the selectivity of these small molecules remains low.[1,2] Moreover, numerous breakthroughs in basic biological research and the unprecedented investment in pharmaceutical research were not sufficient to induce a growing-up drug release on the market.[3] Many very promising bioactive molecules that were recently discovered, as small interfering RNAs (siRNAs), could have tremendous therapeutic applications,[4] but, like others biomacromolecules, the poor bioavailability and rapid degradation in the bloodstream hamper their therapeutic application. The difficulty to cross the plasma membrane and/or the blood-brain barrier (BBB) to reach their target is also an issue. To transform siRNAs into a drug requires developing efficient delivery strategies to fill the gap between benches and beds.

Due to their intrinsic gene delivery ability, viral vectors were developed up to clinical trials. But, the attractiveness of such delivery agents has decreased after several accidents.[5] Safer gene delivery strategies still need to be developed. For *in vitro* use, a large diversity of cationic lipids has been used. They share common features: a headgroup, made of several charged nitrogen atoms, is associated *via* a spacer to long hydrocarbon chains (from 14 carbon atoms). The charged nitrogen atoms promote electrostatic interactions with the anionic phosphate groups of the oligonucleotide while the spacer might have a non-negligible effect (organization, size, hydrophobic interactions with the oligonucleotide). These cationic lipids are formulated with a neutral co-lipid to form unilamellar vesicles, which form lipoplexes when mixed with the oligonucleotide to be delivered. One of this formulation, named LipofectAMINE® (Figure 1 A) is widely used for *in vitro* transfection.[6] High transfection efficiencies have been reported, but many cell types remain difficult to transfect (primary cultured cells) and the cell viability is often reduced after the transfection step. The efficiency of delivery has to be improved, both for *in vitro* and *in vivo* applications.

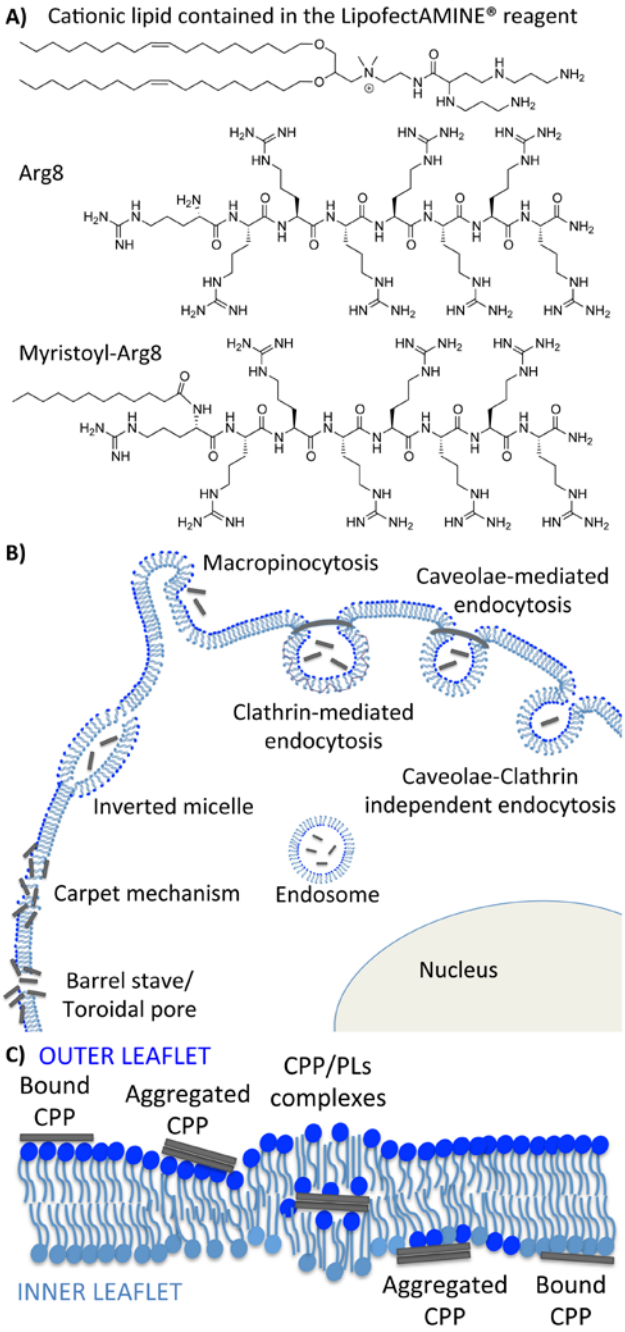


Figure 1. Chemical structures of cationic cell-penetrating molecules and insights into the internalization mechanisms of cell penetrating peptides. A) Chemical structure of a cationic lipid used in the LipofectAMINE® formulation (top), sequence of a polyarginine CPP (Arg8, middle) and formula of this CPP *N*-acylated by myristic acid (bottom). B) Depending on the nature of the CPP and its concentration as well as the cell line and incubation condition, the internalization of the CPP and of its eventual cargo proceed *via* different pathways. The direct translocation corresponds to the spontaneous penetration of the CPP inside the cytosol. Several mechanisms have been proposed to explain how polycationic molecules can cross the plasma membrane (formation of pores, the carpet mechanism, transitory formation of inverted micelles inside the bilayer, on the left). The pathways mediated by the cell machinery (macropinocytosis and

endocytosis, on the right) confine the CPP inside endosomes, where CPP have to escape. C) Model of CPP direct translocation *via* the formation of inverted micelles.[7,8] In this model, CPPs are internalized as neutral and hydrophobic complexes with anionic phospholipids [$CPP^{p+}(PL^{-})_p$]. We further demonstrated that CPPs have to dimerize prior to the formation of the inverted micelle.

The first cell penetrating peptides (CPPs) that were discovered, Penetratin and Tat, are short natural cationic sequences that are spontaneously able to enhance the penetration of proteins into living cells (Table 1).[9,10] Soon after their identification, they have been used to promote the penetration of various exogenous cargoes through cell membranes, as small molecules, peptides, proteins, oligonucleotides and even nanoparticles.[11] Interestingly, they exhibit a low cytotoxicity and immunogenicity. It is now widely accepted that these peptides enter both by energy-dependent (endocytosis, macropinocytosis) and energy-independent pathways (Figure 1B).[12] The role of phospholipids (PLs) in the direct translocation mechanism was soon underscored and it was hypothesized already in 1996, by Chassaing, Prochiantz *et al.*, that CPPs translocate through cell-membranes as inverted micelles.[7] A very similar interpretation, named "mutual adaption" has been made by Wender *et al.*[13] In both models, hydrophilic cationic CPPs recruit the anionic PLs on the outer leaflet of the membrane bilayer. With these PLs, CPPs could transiently form a more lipophilic [(CPPs)(PLs)] complex, which can diffuse through the hydrophobic core of the membrane and deliver the CPPs to the inner leaflet of the membrane. The dissociation of the labile translocation complex delivers the CPP and its eventual cargo to the cytosol. (Figure 1 C).[8,14]

Table 1. 5 CPPs presented herein. Some of their characteristics are indicated: length, net charge, molecular weight and amphipathicity.

CPP	Sequence	Length	Net charge	Molecular weight ($g.mol^{-1}$)	Amphipathicity	Ref
Penetratin	RQIKIWFQNRRMKWKK	16	+ 7	2247	Secondary	[9]
Tat	GRKKRRQRRRPPQ	13	+ 8	1719	Non-amphipathic	[10]
Arg9	RRRRRRRRR	9	+ 9	1423	Non-amphipathic	[15]
TP10	AGYLLGKINLKALAALAKKIL	21	+ 4	2183	Primary	[16]
Transportan	GWTLNSAGYLLGPINLKALAALAKKIL	27	+ 4	2846	Primary	[17]

Since the pioneer discoveries of Penetratin and Tat, the size of the family of CPPs never stopped growing. Over the 15 past years, advances have been made in uncovering and designing new penetrating sequences. For recent reviews on the development of the field of CPPs,[11,18–21] their internalization mechanisms,[14,22–24] and pharmaceutical applications,[25,26] see the indicated references.

Synthetic cationic penetrating sequence have been developed as the octa- or nonaarginine CPPs (Arg8 or Arg9, Figure 1 A and Table 1), directly inspired from the sequence of Tat, or TP10, which is a deletion analogue of the chimeric peptide Transportan (Table 1).[15,16] Cationic CPPs can efficiently deliver both *in vitro* and *in vivo* a broad diversity of bioactive cargoes across cell membranes and also across the BBB. For *in vivo* applications their enantiomers might be used to enhance the plasmatic half-life. Nevertheless, they still require several improvements to be widely applicable. In particular, the direct translocation pathway and/or the endosomal escape has(have) to be enhanced in order to successfully deliver most the cargo to the cytosolic or nuclear target. Moreover, CPPs often partially loose their delivery properties in the presence of serum or plasma.

Many hydrocarbon moieties have been covalently or non-covalently associated to the most common CPPs. These hydrophobic entities dramatically change the physico-chemical properties of the cationic CPPs. In particular, their amphipathicity is profoundly transformed. Tat and oligoarginines are non-amphipathic and Penetratin is a secondary amphipathic peptide (in an α-helical conformation, Penetratin display one hydrophobic side and one cationic side, Table 1). Upon addition of a hydrophobic counterpart, these CPPs become primary amphipathic molecules (their primary structures contain well-defined cationic and hydrophobic domains) or even hydrophobic (in a water/chloroform biphasic mixture, they partition into chloroform). Turning non-amphipathic or secondary amphipathic peptides into primary amphipathic or hydrophobic molecules increases their affinity for neutral or weakly anionic membranes bilayers because they can interact both with the phosphate groups of the phospholipids and with the hydrophobic core of the membrane bilayer.[14,27] This property is particularly interesting to enhance the local concentration of cargoes at the plasma membrane of eukaryotic cells and thus to favor their uptake by active transport. Changes in the amphiphilicity of the CPPs will be discussed in more details all along this review.

Very interestingly, the addition of a hydrophobic moiety can also change the main internalization pathway of CPPs and enhance the direct translocation or the endosomal release of the cargo conjugated to the modified CPP. This is not surprising given the possible participation of these hydrophobic counterparts to the formation of inverted micelles (Figure 1 C).

Besides the internalization of cargoes, the acylation of CPPs with long fatty acids presents also several advantages: it enhances the formation of stable CPP-oligonucleotides complexes, reduces their sensitivity to serum and protects oligonucleotides from enzymatic hydrolysis. The *in vitro* transfection efficiencies of some acylated CPPs exceed those of the commercially available transfection reagents as LipofectAMINE®. The *in vivo* oligonucleotide delivery has been also improved.

The introduction of hydrocarbon moieties has opened new avenues in the design of vectors that may be used for drug targeting. We will review the various strategies, which have been developed to improve the delivery properties of CPPs. Most importantly, we will discuss their benefit for the delivery of small molecules, oligonucleotides and nanoparticles, *in vitro* and *in vivo*.

1. Enhancing the translocation properties of oligoarginine cell-penetrating peptides with hydrophobic counteranions

Anionic phospholipids are natural activators of the direct translocation of arginine-rich CPPs through membranes. They interact both electrostatically and by forming hydrogen bonds with the guanidinium moieties. Exogenous counteranions could specifically enhance the direct translocation pathway by temporarily reversing hydrophilic of oligoarginines into hydrophobic molecules. Sakai, Futaki, Matile and their coworkers tackled this fundamental question, both to understand the penetration mechanisms and to enhance the penetration of the usual CPPs.[28]

1.1 Amphiphilic counteranions promote the internalization of arginine-rich CPPs

By using the pyrenebutyrate counteranion (PyB, Figure 2 A), they first succeeded in increasing the internalization of the fluorescently labeled octaarginine CPP (Arg8). This improvement in cell penetration depends on the concentration of the added counteranion.[29,30] In a typical experiment, cells are washed several times with phosphate-buffered saline (PBS) prior to a 5-min pre-incubation of the cells with PyB (50 μM) in PBS. The arginine-rich CPPs are then added to the desired final concentration (about 10 μM) and co-

incubated in the presence of PyB. The incubation has to proceed in PBS to avoid any exchange between PyB and the other counteranions, which could be present in the culture medium.[30]

Whereas a cell population is usually inhomogeneously stained by fluorescently-labeled CPPs, a homogeneous labeling of the population is obtained if the cells were pre-incubated with PyB.[30,31] Moreover, in the presence of PyB, internalization proceeds very quickly (10 min) without neither apparent cytotoxicity nor membrane permeabilization, yielding to a uniform fluorescence. This concept of enhancing penetration by adding an amphiphilic counteranion is widely applicable to the other well-known arginine-rich CPPs (Penetratin and Tat) and on multiple cell lines.[30,32]

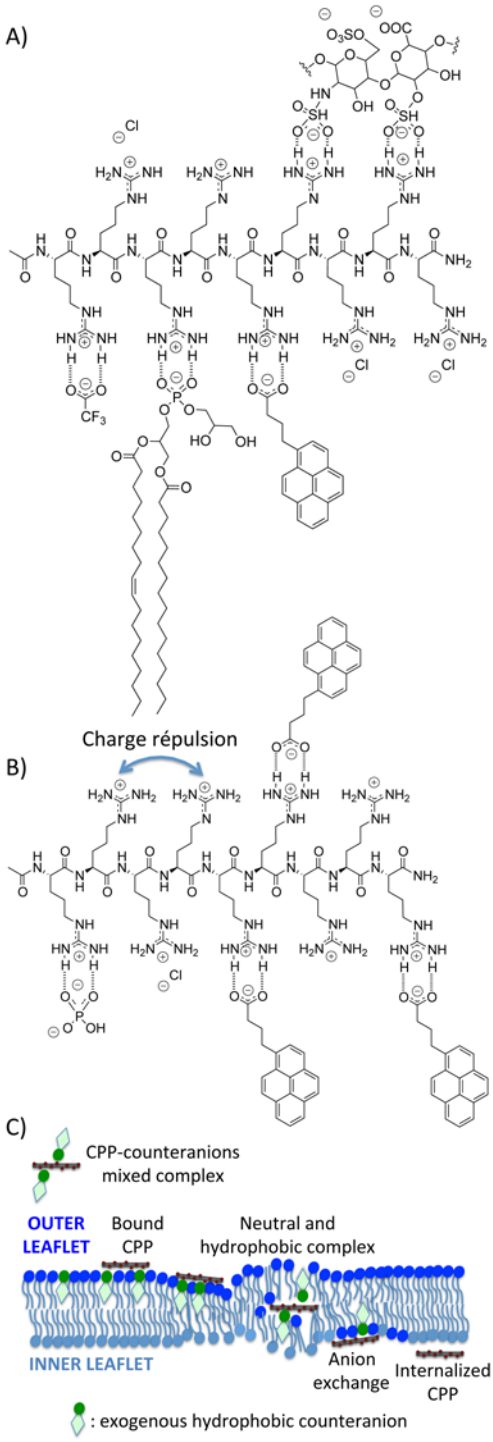


Figure 2. Origin of the interaction between exogenous hydrophobic counteranions and polyarginines and their role in the direct translocation mechanism. A) Some counteranions that have been used and their interaction with the guanidinium group. B) The particular affinity of oligoarginines for counteranions is intrinsically related to their polycationic nature and their high content in residues, which are not acidic enough to be deprotonated at physiological pH (the pKa of arginine is 12.5; the pKa of a lysine is 10.5, but for electrostatic repulsion oligolysines would be only partially protonated). To minimize the charge repulsion, binding energy of arginine side-chain and anions will be much stronger than for isolated

arginines.[33] As a consequence, these polyarginines will always be surrounded by counteranions. C) Direct translocation in the presence of PyB. The counteranion might participate to the translocation transition state and enhance the rate of its formation.

1.2. Amphiphilic counteranions increase the transporter function of CPPs

As a proof of concept, the delivery of enhanced green fluorescent protein (GFP, 30 kDa) and of ^{15}N-labeled ubiquitin (9 kDa) respectively fused with Arg8 and Tat were analyzed.[30,34] Both proteins were successfully internalized into HeLa cells in the presence of PyB, yielding to a diffuse cytosolic fluorescence. The uniformly ^{15}N-labeled Tat-ubiquitin fusion protein was delivered in a sufficient large amount to HeLa cells to be used for in-cell high-resolution NMR of protein (meaning that 20-30 μM concentrations were reached).[34]

The delivery of bioactive compounds is much more demanding because it requires to reach a specific target. The effect of the counteranion-mediated delivery of CPP-bioactive cargo has been analyzed for the delivery of the pro-apoptotic domain peptide (PAD peptide) and for a splice-switching oligonucleotide.[30,32] The pre-incubation of the cells with PyB increases dramatically the pro-apoptotic effect of the Arg8-PAD conjugate as deduced from the depolarization of mitochondria and the reduced cell-viability.[30]

1.3. The mechanisms of PyB-mediated enhanced CPP and CPP-cargo intracellular delivery

Several arguments support the PyB-mediated increase of direct translocation. Firstly, confocal microcopy shows that the majority of the fluorescence associated to the labeled peptide is diffuse. Secondly, the subcellular distribution and the amount of internalized fluorescent CPP are similar at 4 °C (when all energy-dependent internalization mechanisms are diminished). Comparatively, in the absence of PyB and at 4 °C, no intracellular localization was noticeable, and at 37 °C the fluorescence of the CPP is punctuated (*ie.* localized in endosomes or lysosomes).[30] Finally, the internalization is quite suppressed if the membrane potential is reduced by incubation in a PBS buffer where sodium is substituted by potassium. Under such experimental condition, endocytosis is not inhibited.[13] As a conclusion, direct translocation is the favored internalization pathway for PyB-mediated oligoarginine and its driving force is the transbilayer potential.

Some aspects of the PyB-mediated translocation pathway have been recently investigated using model membranes. A homogenization of the extra- and intravesicular concentration of Arg8 through the membrane

of anionic GUVs (giant unilamellar vesicles) was directly observed with confocal microscopy (DOPC/DOPS/SM/Cholesterol, 1:1:2:1). In the presence or absence of Arg8, PyB induces the disruption of liquid ordered domains (L_o), thus increasing the overall fluidity of the membrane. This increase in membrane fluidity might be responsible for the larger translocation ability of oligoarginines,[35] and PyB might participate to the translocation transition-state in fluid membranes as an anionic phospholipid would do (Figure 2 B).

1.4. PyB-mediated CPP translocation has been first proposed and investigated using membrane models

It is fascinating to realize that this concept was first investigated with bulk membranes made of chloroform and artificial membrane bilayers before being applied to living cells. This is one important success of the bottom-up approach using model membranes.

The enhancement of lipophilicity of oligoarginines in the presence of amphiphilic counteranions, such as egg yolk phosphatidylglycerol or PyB, was ascertained by the increased transfer of oligoarginines into chloroform (Figure 2 A).[33,36] Interestingly, it was also observed that polysulfated polysaccharides, such as heparin, inhibit this transfer, showing that a competition between counteranions may exist (Figure 2 A). This information is relevant due to the presence of complex sulfated polysaccharides on cell surface (the glycosaminoglycans).

The translocation of oligoarginines does not correspond solely to a transfer of the peptide from the aqueous phase to the hydrophobic core of the membrane, but to a phase transfer followed by a reverse phase transfer from chloroform to the aqueous phase. The adaptability of the oligoarginines environment was monitored as the transfer across bulk membranes. This experiment enlightened the role of hydrophilic counteranions, which are able to exchange rapidly with the lipophilic counteranion, allowing a successful transfer *across* the bulk membrane. Such “activator” counteranions would promote the direct translocation pathway. The association between oligolysines and similar counteranions would be much weaker because i) all the lysine residues are not protonated at physiological pH, and ii) the absence of the bidentate hydrogen bonding observed with the guanidinium side-chain (Figure 2 A). Among the various counteranions that have been tested, PyB turns to be the most successful activator of translocation on living cells.[28,29]

The counteranion-mediated translocation enhancement has one major drawback: it requires a serum-free medium and is thus not applicable to enhance *in vivo* drug delivery. Nevertheless, hydrophobic counteranions remain very useful for the *in vitro* delivery of small peptides as well as large proteins and might be used as adjuvants.

2. Lipidated CPPs as versatile vectors

Myristoylation and palmitoylation of proteins are natural post-translational modifications, which enhance membrane association of cytoplasmic proteins.[37,38] In pharmacology, the addition of a lipid anchor is a general strategy that is currently in use to concentrate on cell surface peptides targeting membrane proteins.[39,40] Moreover, it was found in the early 1990s that the myristoylation of some basic peptides enhanced their penetration.[41,42] Since then, this strategy has been flourishing.[43,44] Interestingly, the enhanced thermodynamic stability of oligonucleotide-lipopeptide complexes was already reported with acylated antimicrobial peptides, as well as the non-covalent successful delivery of oligonucleotides.[45,46]

The mechanisms of cell internalization of acylated peptides and the physico-chemical properties required for the acyl chain (quantity, in-cell distribution, low membrane perturbation) are still rather vague and need to be refined. But, it was ascertained that the presence of both cationic residues and a fatty acid moiety are necessary for the internalization.[47] Acylation of a neutral peptide does not increase its cellular uptake.[48] Even if acylation or farnesylation of a peptide is not sufficient to yield a stable anchoring to a membrane, the presence of both a cationic domain and an acyl chain cooperatively promotes membrane affinity.[49,50]

The acylation of the usual CPPs at their *N*-terminus can be easily performed on solid support; only the acylation with acid-sensitive hydrocarbons remains problematic because of the acidic cleavage conditions. We will briefly review the synthesis procedures of CPPs modified with an acyl chain, before focusing on the successful use of lipopeptides for both *in vitro* and *in vivo* delivery of various cargoes. We will systematically discuss the internalization mechanisms of hydrocarbon-enhanced CPP drug delivery.

2.1. General strategies for the lipidation of CPPs

The *N*-terminal addition of a saturated fatty acid moiety is the most frequent derivatization (Figure 3 A top and middle). Two protocols have been used for the solid-supported *N*-terminal addition of saturated fatty

acids: direct addition on the *N*-terminus *via* the formation of an amide bond (various coupling agents can be used) or after a short β-alanine linker.[51–56]

The direct introduction on solid support of an unsaturated hydrocarbon chain is impossible with Boc solid phase peptide synthesis (SPPS) because of the hydrofluoric acid treatment. However, Fmoc SPPS is less efficient for the synthesis of oligoarginines, Penetratin and Tat CPPs (double coupling steps and microwave-assisted SPPS are often required) (Swiecicki, unpublished results). And even the final deprotection with trifluoroacetic acid remains problematic in the presence of acid-sensitive unsaturated chains. Thus, the addition of unsaturated chains must be performed in solution. In the absence of a lysine residue, cholesterol was reacted as cholesteryl chloroformate with the free *N*-terminal amine of oligoarginines in the presence of triethylamine (Figure 3 A, bottom).[57,58] Farnesylation or geranylation was also performed in solution. A free cysteine residue of the cargo may be alkylated with farnesyl or geranyl bromide in the presence of a Zn(II) catalyst prior to the addition of the CPP.[59] Cationic peptides whose *N*-terminus has been acylated with an hydrophobic moiety are members of the "linear peptides amphiphiles" family (LPAs).

The introduction of such a hydrophobic chain dramatically perturbs the physico-chemical properties of the parent CPPs. In particular, non-amphipathic oligoarginines become primary amphipathic LPAs. The resulting consequences on the internalization have been investigated and are the object of the next sections.

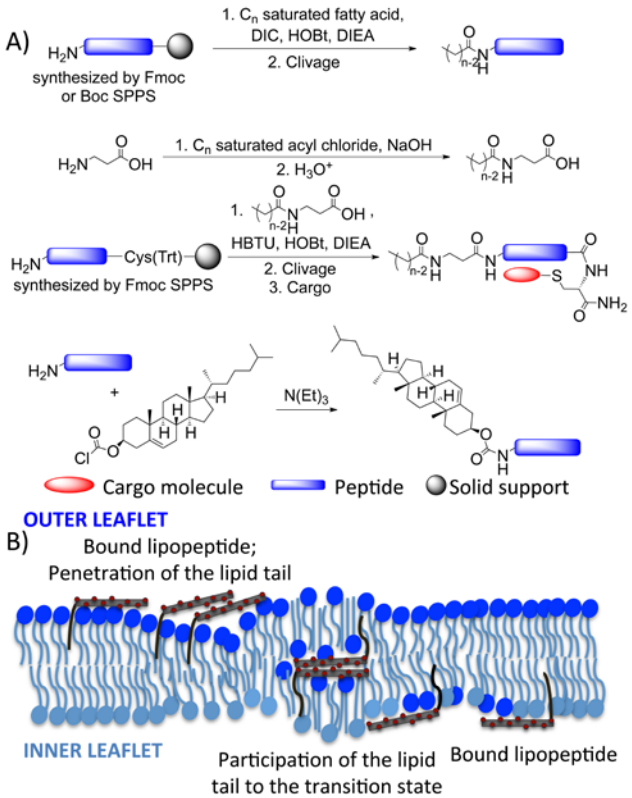


Figure 3. Strategies to introduce a hydrocarbon moiety to the *N*-terminus of a CPP and its potential role during direct translocation. A) The *N*-terminal introduction of a saturated hydrocarbon moiety can be directly performed on solid support (top). Alternatively a modified Schotten-Baumann reaction can be performed in solution followed by an amide bond formation (middle).[55] A stable carbamate bond is formed, when cholesterol was reacted as cholesteryl chloroformate with the *N*-terminal amine in solution (bottom).[57] B) The *N*-terminal acyl chain might participate to the translocation transition state and enhance the rate of this spontaneous internalization pathway.

2.2. Towards the internalization mechanism(s) of lipidated oligoarginines

Oligoarginine CPPs have a very high affinity for surface glycosaminoglycans, but a lower affinity for the outer leaflet of eukaryotic membranes.[27] In the first reports on the derivatizations of oligoarginines with hydrocarbon moieties, it was clearly stated that the acylation must enhance membrane affinity, and subsequently the internalization ability.[54] Indeed, the primary amphipathic character induced by the addition of a lipid tail to non-amphipathic or secondary amphipathic CPPs favors the association of the CPP to the membrane. This increase in cellular association might result in an increase of the endocytic uptake. But, the acyl chain might also trigger direct translocation, by participating and eventually facilitating the direct translocation transition state: the inverted micelle (Figure 3 B).

The structures of the oligoarginine LPAs that were synthesized for the mechanistic studies are similar: a fatty acid covalently associated to the *N*-terminus of the peptide and a fluorophore coupled to the side-chain of the *C*-terminal amino acid. The influence of the number of arginines and the fatty acid chain length were both investigated. The affinity of the LPAs for the plasma membrane generally increases with the number of arginines and with the acyl chain length, the association with cell surface being 10 times larger for myristoyl-Arg11 than for Arg7 or Tat CPPs.[54] The influence of these parameters on the internalization are more difficult to rationalize, and it is impossible to reach an evident trend. Nevertheless, it is clear that the acylation of oligoarginine CPPs increases dramatically their penetration ability into different cell lines, [48] even a short acyl chain (hexanoyl) enhances the internalization.[52] If most of the lipopeptides do not affect cell viability, LPA with long acyl chains and especially long oligoarginines (13 or 15 arginine residues) are slightly cytotoxic.[48,48,52]

At a concentration below 10 μM, most of the cells incubated with fluorescent Arg8 or Tat CPPs exhibit a punctate fluorescence, which is attributed to a confinement of the CPPs inside endosomes and thus to energy-dependent uptake mechanisms. In contrast, the fluorescence of acylated oligoarginines with short acyl chains is more diffuse in the cytosol, being also present in the nucleus (short chains = myristoyl chains and shorter).[48,52] However, punctate fluorescence is still observed. A co-localization with subcellular compartment such as the Golgi apparatus and the reticulum has also been proposed, related to the high affinity of LPA for membranes.[54] The fluorescence between the different cells is also not homogeneous, in contrast with the observations made with the PyB counteranion strategy. This heterogeneous cell internalization was interpreted for Tat and Arg9 as a threshold effect.[31] More detailed insights into the internalization mechanisms were obtained using energy-dependent inhibitors (chemicals or temperature). Oligoarginine CPPs, which are acylated by short chains, enter by both energy-dependent mechanisms and direct translocation, and both translocation pathways are enhanced in the presence of a fatty acid chain.[48,52] Arg8 acylated with stearic acid enters *via* an energy-dependent pathway, as deduced from the low internalization at 4 °C,[60] probably related to the formation of large aggregates due to its low critical micelle concentration (CMC).[61]

The number of arginine residues and the length of the acyl chain need to be optimized, depending on multiple factors such as cell line, cargo and targeted compartment. Nevertheless, acylated oligoarginines with the adjusted properties are promising delivery vectors, which have been successfully used for the *in vitro* and *in vivo* delivery of small molecules, proteins, and even nanoparticles.

2.3. Acylated oligoarginines as covalent small molecules, proteins and nanoparticles delivery vectors

Futaki *et al*. probed the delivery efficiency of these acylated oligoarginines. The PAD peptide, the small protein ubiquitin (9 kDa) or the larger carbonic anhydrase (29 kDa) were conjugated to the *C*-terminal cysteine side-chain of hexanoyl-Arg8 LPA.[52] The deliveries of these cargoes were increased in comparison to the one of the Arg8 conjugates, as estimated by confocal microscopy or by cell viability assay (proapoptiotic effect of PAD). This internalization enhancement cannot be translated to every cell type, meaning that this LPA requires further optimization for systemic delivery. Alternatively, its specificity might also be improved to target certain cell types.

Moore *et al.* investigated, after *i.v.* injection, the distribution of myristoylated-Arg7 LPA labeled with a near infrared fluorophore. Imaging of whole nude mice and then of the excised brain revealed that this lipopeptide can progressively deliver the fluorescent probe to the brain, in contrast to the fluorophore alone (from 6 to 44 hours after the injection).[55] The fluorescence is related, at least in a large part, to the intact myristoylated-Arg7 labeled LPA, which is distributed in the whole brain, and can thus not only penetrate cells but also cross the BBB. With the same lipopeptide, Moore *et al.* modified the surface of superparamagnetic iron oxide nanoparticles for MRI imaging.[62,63] These nanoparticles were not only used as a contrast agent but also as a platform for a near infrared fluorophore for imaging and siRNA delivery. The siRNA that were covalently linked to the surface target GFP mRNA. These nanoparticles were injected *i.v.* to mice that were grafted with tumors expressing either GFP or RFP (red fluorescent protein). Myristoylated-Arg7 accumulates the whole nanoparticles inside tumors, as demonstrated by MRI and near infrared imaging, and specific GFP and not RFP silencing was observed, demonstrating the successful cytoplasmic siRNA delivery.[63] Similarly, modified iron oxide nanoparticles are able to cross the BBB and accumulate into a brain tumor, only in the presence of this LPA. Tumor detection by MRI was subsequently facilitated with this non-invasive protocol.[62]

In summary, the cargo-lipopeptide covalent strategy offers the possibility to deliver efficiently *in vitro* and *in vivo* various cargoes. With this strategy, the conjugates have a well-defined chemical structure, which allows an accurate knowledge of the chemical specie and a finer rationalization of the internalization mechanisms. But, this strategy is time-consuming and cost-ineffective due to the modifications that need to be introduced on the chemical structures of the cargoes (*i.e.* preparation of fused proteins or derivatizations of oligonucleotides). Thus, a non-covalent delivery based on the primary amphipathic character of acylated CPPs might be a very attractive alternative.

2.4. Acylated oligoarginines supramolecular assemblies as non-covalent small-molecules vectors

Dehsorkhi *et al.* recently published a very detailed review describing the great variety of nanostructures that can be obtained starting from self-assembling LPAs.[64] The nature of the hydrophobic core of a LPA governs size, shape, and aggregation of the self-assembled peptide-based nanostructures. By introducing different lipid structures it is possible to finely tune the properties of the supramolecular aggregates.

The self-assembly properties of a novel class of supramolecular building blocks in which the CPP Tat and a lipid dendrimer (possessing from 2 to 8 stearyl arms) are covalently bound were investigated by Lim *et al*. The morphology of such Tat-based dendrimers is mostly driven by the hydrophobic interactions of the lipid chains. Monostearylated Tat does not self assemble, even at high concentration (1 mM). Distearylated Tat forms spherical micelles (CMC = 208 μM), while tetrastearylated Tat self-assembles mostly in short-length cylindrical micelles (CMC about 21 μM). These short-length cylindrical micelles (nanorods with an average length of ~ 100 nm) could be highly loaded (~ 4 mol%). Tetrastearylated Tat nanorods do not disintegrate when interacting with plasma membrane, but maintain their self-assembled state during the cell internalization process.[65] As proof-of-concept it has been demonstrated that these nanorods are internalized in HeLa cells delivering the encapsulated Nile Red cargo even in the nucleus. Cylindrical micelles can be also obtained with Tat derivatized with either four or eight octanoic chains.[65,66] These nanofibers can efficiently encapsulate the hydrophobic anticancer drug paclitaxel (PTX). Highly PTX-loaded nanofibers (~ 7 mol%) are flexible structures that are actively internalized by an absorptive-mediated pathway. Zhang *et al.* proposed an endocytotic uptake of drug-loaded nanofibers in which the encapsulated drugs can diffuse out of the lysosomes only after disassembly of the nanofibers. Indeed, PTX-loaded nanofibers deliver PTX into KB-3-1 cervical cancer cells, without reducing PTX efficiency.[66]

Chen *et al.* designed a tetra-tail stearylated octaarginine peptide incorporating at the *C*-terminus the RGD sequence, as the RGD sequence improved cancer cells targeting. This PA self-assembles in 200 nm-size micelles (CMC = 5 μM). Doxorubicin was encapsulated into these micelles and its antitumor activity was improved with reduced side effects.[67]

2.5. Acylated oligoarginines as non-covalent oligonucleotide vectors

Due to the high density of cationic residues, oligoarginine CPPs can bind and compact DNA. Complexes between Arg8 to Arg15 CPPs and DNA were prepared by mixing both entities and good transfection efficiencies were achieved.[51,68] Nevertheless, complexes between short basic peptides and DNA are not very stable and an important amount of the complex remains trapped inside endosomes.[57,69,70] The acylation strategy circumvents the weakness of small peptides-oligonucleotides complexes.[46]

N-terminal addition of fatty acids or cholesterol dramatically enhances the transfection efficiency of plasmid DNA compared to Arg8, as demonstrated by Futaki *et al*.[51] The most efficient LPA is stearyl-Arg8, whose transfection capability reaches the same order of magnitude than the one obtained with LipofectAMINE®. The uptake of stearyl-Arg8-DNA complexes proceeds essentially, as expected, *via* endocytosis, given their size (~100 nm).[60] The difference in transfection efficiency between stearyl-Arg8 and Arg8 has been interpreted as a difference in DNA compaction associated to: (i) the aggregation of the alkyl chains, and (ii) the enhancement of membrane interaction with the high number of alkyl chains presents in the stearyl-Arg8-DNA complex, both effects promoting endocytosis. Fluorescence confocal microscopy showed that the amount of internalized DNA was effectively increased in the presence of stearyl-Arg8 and that some of the DNA localized into the nucleus.[60]

Stearyl-Arg8, as well as myristoyl-Arg7 and cholesteryl-Arg9, also enhances the uptake of siRNA.[57,71,72] Interestingly, siRNA form stables complexes with lipopeptides that protect them from nucleases-mediated hydrolysis, and even *in vivo* delivery of intact siRNA has been observed.[71,72]

2.6. Acylated TP10 as non-covalent oligonucleotide vectors

Langel *et al.* developed the same strategy for TP10, and then various generations of acylated TP10 under the generic name "PepFect", to progressively improve the transfection properties of TP10. The last generations of PepFect lipopeptides are much more efficient than LipofectAMINE®.

TP10-oligonucleotide complexes are internalized, but the oligonucleotides remain trapped inside endosomes and chloroquine is required to enhance their endosomal escape.[53,56] However, because of its toxicity, chloroquine cannot be used at high concentration for systemic application. The first modification that was introduced is the addition of a stearyl acyl chain at the *N*-terminus of TP10 (named PepFect3). This modification increases the internalization of a splice-correction oligonucleotide. But the addition of chloroquine again enhances the oligonucleotide delivery, demonstrating that a non-negligible fraction of oligonucleotide remains trapped into the endosomes.[53]

PepFect6 is the next generation, with a lysine side-chain modified by a small dendrimeric structure of chloroquine derivatives whose role is to enhance endosomal escape without the need of adding chloroquine. Stable PepFect6-siRNA complexes promote the *in vitro* and *in vivo* delivery of siRNA followed by the

desired gene silencing in various organs.[73] The non-covalent delivery properties were enhanced by finely tuning the amino acid side-chains of PepFect3 to afford PepFect15.[74,75] This lipopeptide is twice as efficient as LipofectAMINE® with no apparent cytotoxicity. Interestingly, this family of lipopeptides does not exhibit *in vitro* toxicity nor immunogenicity.[76]

Lipidation of the existing CPPs is a promising strategy that allows some tuning of their initial properties. Depending on the acyl chain length, direct translocation and/or energy-dependent internalization mechanisms can be enhanced. Most importantly, the delivery of various cargoes covalently or non-covalently associated to the lipidated CPP was increased. This strategy has even been successfully applied to *in vivo* oligonucleotides delivery via non-covalent LPA-oligonucleotides complexes. These complexes are stable enough to circulate in the bloodstream and to preserve the oligonucleotides from nuclease activity but also sufficiently labile to release oligonucleotides in the cytosol or the nucleus.

However, LPAs have some drawbacks. Because of their amphiphilic nature, their HPLC purification may be challenging. Their solubility may be low resulting in unstable concentrated stock solution. Moreover, an optimization of both the CPP and the fatty acid moiety has to be performed depending on the physico-chemical properties of the cargo and on the subcellular localization of the target. Up to now as for CPP, "predictive properties" remains The issue. As a consequence, it might be interesting to design minimalist lipopeptides, whose synthesis can be performed in a few steps on solid support or even in solution.

3. Modification of the structure of acylated arginine-rich CPPs. *En route* for minimalist Trojan carriers

Enhancing the hydrophobic contribution of the interaction between CPPs and the plasma membrane can induce passive uptake, probably by increasing the association of the peptide with the membrane *via* the PLs tail. Understanding how the balance of hydrophobic and electrostatic interactions controls CPP-membrane interactions and, hence, membrane translocation may be the clue for designing new CPPs with specific membrane activity.[77]

In the following sections, different structures of acylated cationic peptides will be presented and their related applications will be discussed.

3.1. Short cationic peptides amphiphiles

The chemical, physical, and biological properties of short cationic LPAs can be controlled by manipulating the chain length of the peptidic backbones, the amino acids sequence and the nature of the hydrocarbon moiety. The minimization of cationic domains can be a straightforward strategy to identify key functional groups in CPP and hence to design small(er) size peptides possessing improved delivery efficacy. Reducing the carrier size would simplify their synthetic access; even thought solubility requirements have to be kept in mind as soon as hydrophobic counterparts are introduced.

It has been demonstrated that decreasing the number of guanidinium moieties in linear CPP carriers (based on a bis-ornithine scaffold) does not hamper efficient cargo delivery. Myristoylation of cationic peptides enhances cellular uptake in a peptide-sequence depending fashion, with a great effect on Arg9, but not on Penetratin and Knotted (two peptides derived from the third helix of homeodomains).[78] Burlina *et al.* designed and evaluated the translocation properties of a serie of amphipathic CPPs sharing a minimized tetraarginine cationic domain and characterized by different lipophilic chains (Figure 4 A).[61] They found that amphipathic tetraarginine CPPs acylated with a long alkyl chain were more efficient carriers of a peptidic cargo than Arg9 ("long alkyl chain" = 12 carbons and higher). Noteworthy, in CHO-K1 cells, direct translocation across the plasma membrane turn to be a non-negligible pathway, with the GAG-dependent pathway remaining the major internalization route. This correlates with their tendency to aggregate in solution.[61]

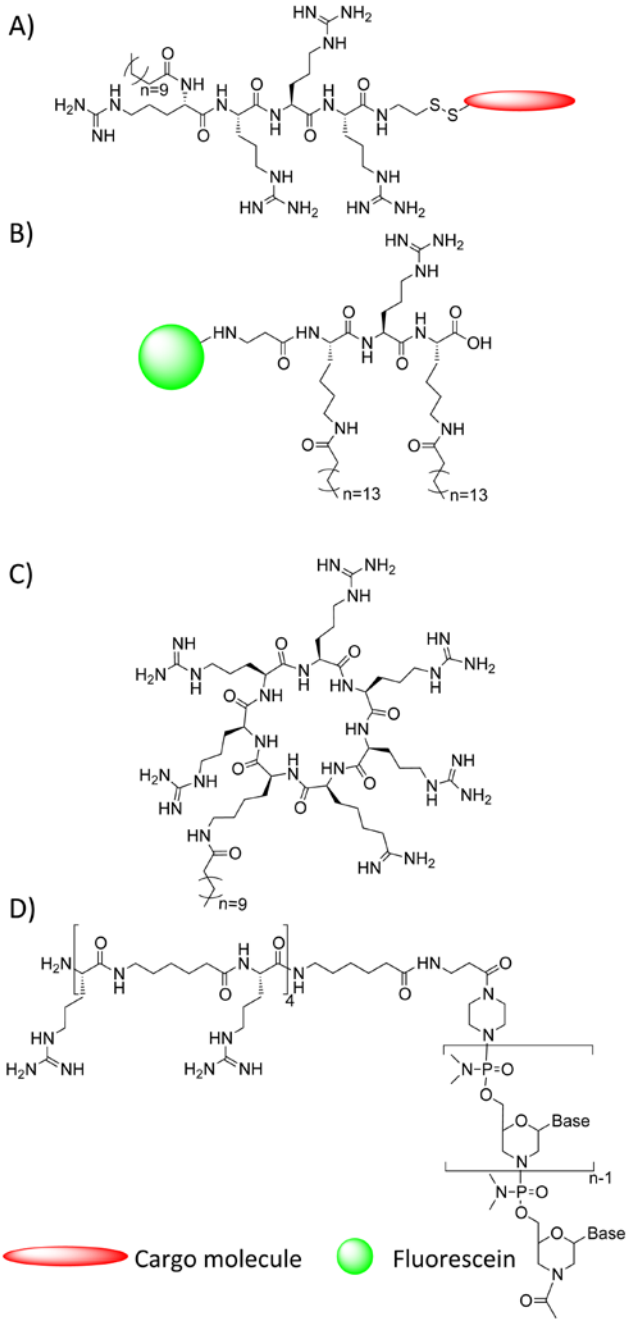


Figure 4. Four examples of short cationic LPAs. A) The lipopeptide stearyl-tetraarginine has been studied by Burlina *et al.* for the covalent delivery of small cationic peptides.[61] B) Short cationic LPA containing one arginine and two lysines conjugated with palmitic acid were developed by Shirazi *et al.*[79] C) Oh *et al.* prepared a cyclic dodecanoyl-[Arg6] LPA for the non-covalent delivery of small anionic phosphopeptides.[80] D) Structure of the covalent conjugate $(Arg\text{-}Ahx\text{-}Arg)_4$-PMO described by Abes *et al.* as a tool to redirect the splicing machinery.[81]

Fujita *et al.* demonstrated that complexes formed by short basic domain made of tetraarginine conjugated to PEG lipid-coated and complexes with DNA and protamine possess high transfection efficiency in tumor

cells.[82] Shirazi *et al.* evaluated different short polycationic LPAs containing lysine(s) and arginine(s) acylated by fatty acyl groups (Figure 4 B). They showed that a LPA containing one arginine and two lysines acylated by two palmitoyl acyl chains was able deliver into the cytoplasm a fluorescently labeled drug and a phosphopeptide. The endocytotic traffic was hypothesized to be the major internalization pathway.[79]

Cationic lipids bearing lysine, arginine or histidine headgroups have been explored and used for plasmid DNA delivery and gene transfection. Interestingly, these LPAs self-assemble into large unilamellar vesicles (LUVs, diameter ~ 100 nm), in analogy with lipoplexes containing negatively charged nucleic acids. Obata *et al*. found that the lysine and arginine-type lipid formed LUVs, with an increased size for the cationic assembly in the presence of pDNA. But, the histidine-type lipids led to a tube-like morphology. The gene expression efficiency in relation to the cationic headgroup of the lipids was as follow: lysine, arginine and then histidine. Shorter alkyl chains improved the efficiency of gene expression.[83]

Prata *et al*. found that the model tripeptide Lys-Trp-Lys acylated by two fatty acids at its *C*-terminal extremity forms vesicular structures. The presence of membrane bilayers was demonstrated by X-ray diffraction. In NIH 3T3 cells, the dipalmitoylated LPA was the best candidate for non-covalent gene transfection among a series of amphiphilic peptides having the same headgroup and different fatty acid chain lengths.[84]

Cyclisation of the cationic domain has been investigated to increase the proteolytic stability of the lipopeptides. The efficiency of acylated polycationic cyclic penta- and hexapeptides was evaluated. Dodecanoyl-[Arg6], with an acyl chain bound to a Lys residue of the cyclic peptide, was the most potent for shuffling into cells a cell-impermeable phosphopeptide (Figure 4 C). The internalization mechanism was found to be energy-dependent.[80]

Furthermore, the screening of libraries of dynamic amphiphiles as delivery vehicles can be a convenient approach to identify new lipopeptides as siRNA transfecting agents.[85]

3.2. The hydrophobic component can be introduced by using unnatural aminoacids

Arginine-rich CPPs, like $(Arg\text{-}X\text{-}Arg)_n$ peptides, where X is a generic carbon chain spacer, have been designed. The insertion of non-natural aminoacids, such as 6-aminohexanoic acid (Ahx), is expected to

increase the metabolic stability. Wender *et al.* reported that Ahx seems to be optimal spacer for cellular uptake.[86] $(Arg\text{-}Ahx\text{-}Arg)_4$ can deliver phosphoramidate morpholinooligonucleotides (PMO) within HeLa cells, an $(Arg\text{-}Ahx\text{-}Arg)_4$-PMO conjugates (Figure 4 D) led to a high splicing correction efficiency.[81] Similarly, the stearyl-$(Arg\text{-}Ahx\text{-}Arg)_4$ peptide was found to be very efficient to convey antisense splice-correcting oligonucleotide in the HeLa cells.[87]

In the same line, Ye *et al.* designed a new class of cationic tripodal LPAs characterized by the presence of a saturated alkyl spacer on the Lys-X-Arg-X-Arg sequence, with X being either 5-aminopentanoic acid (Ava) or 12-aminododecanoic acid (Ado). Only Lys-Ado-Arg-Ado-Arg, behaves as a non-covalent carrier for phosphopeptides and its cellular uptake occurs *via* an energy-independent mechanism.[88] Later on, they further investigated the binding properties of these tripodal LPA with respect to model membranes to dissect the binding events and the predominant interactions. They found a correlation between the cellular uptake and the extent of the structural changes induced in the bilayer. Lys-Ado-Arg-Ado-Arg binding with DPPC/DPPG (70:30) LUVs is indeed associated with membrane thinning and permeabilization. The membrane leakage induced by Lys-Ado-Arg-Ado-Arg is greater than the one induced by Lys-Ava-Arg-Ava-Arg. This result suggests that the length of the alkyl linkage can control the penetration into the bilayer.[89] Lys-Ado-Arg-Ado-Arg anchors the membrane via favorable hydrophobic interactions and is able to penetrate into the bilayer inducing phase separation, permeabilization, and disruption of the membrane.[90] The success of minimalist Trojan carriers in delivering various cargoes opens new perspective because their structure can be finely tuned. By adjusting the properties of the carrier, virtually all the desired cargoes might be delivered.

4. Acylated oligoarginines mediate the internalization of multifunctional liposome-based delivery platform

Liposomes with an average diameter of 100–200 nm are widely investigated in nanomedicine as drug delivery agents: convenient processes have been successfully developed to efficiently load drugs in their lumen, functionalization of the surface with PEG moieties increased their circulation lifetime in the bloodstream.[91] Similarly as other high molecular weight and long-circulating nanoparticles, they have a high propensity to accumulate in the interstitial space of solid tumors via the so-called “enhanced

permeability and retention effect" (EPR effect).[92,93] High loading and passive targeting improve the drug specificity and efficiency and PEGylated liposomes are now being used clinically.[94]

The modification of the liposomal surface with CPPs as an attractive strategy to exploit the penetration properties of CPPs to improve liposomes uptake will be described. Furthermore, internal trafficking issues will be discussed.

4.1. The concept of multifunctional envelope-type nanodevices

Harashima *et al*. have introduced the concept in 2004 of multifunctional envelope-type nanodevice (MEND, ~300 nm) for the intracellular targeting of plasmid DNA (pDNA), proteins or other molecules. Typically, MENDs consist of a nucleic acid core condensed with a polycation and a lipid envelope resulting in a core/shell structure. This lipid envelope, containing fusogenic lipids to boost endosomal escape (like DOPE), is functionalized with different entities such as, cleavable PEG for longer blood circulation, a ligand for specific targeting and/or a CPP to enhance the intracellular delivery of the cargo. To anchor CPPs to the MENDs surface, it is necessary to modify the CPPs sequences by adding a lipophilic tail, which will interact with the lipid envelope, while the cationic residues decorating the surface of the MEND will interact with the plasma membrane. One of the most commonly used acylated CPP is the stearyl-Arg8.[95]

Herein, we will briefly report the procedure for the preparation of a MEND encapsulating DNA according to what is called a "programmed packaging". It involves a 3 steps process: (i) DNA condensation with a polycationic polymer, such as poly-L-lysine, (ii) hydration of the lipid film formed by the evaporation of a chloroform solution of DOPE and cholesteryl hemisuccinate, (iii) addition of the condensed DNA in solution to the lipid film. This third step corresponds to the formation of the lipid packaging. To encapsulate the condensed core with lipids, the DNA/poly-L-lysine suspension and the hydrated lipid film are sonicated. Finally, for the coating of the lipid-DNA particle with CPP, the stearylated-Arg8 is added as a solution in buffer, and the mixture incubated at room temperature (see Figure 5 for a schematic representation of a MEND).

Kogure *et al*. developed a MEND encapsulating pDNA to be tested as a novel non-viral gene delivery system. They found that the incorporation of stearylated-Arg8 in the lipid envelope confers attractive penetration properties to the liposome and enhances transfection activities as well as low cytotoxicity.[96]

Later on, they developed a MEND, still decorated with stearyl-Arg8, in which the encapsulated siRNA is condensed with the spermatozoal peptide protamine. This MEND was designed to deliver to the nucleus siRNA expression plasmids, as post-transcriptional gene silencing effector. In that example, MENDs encapsulating siRNA condensed with protamine showed a significantly higher silencing effect in transformed COS7 cells than MENDs encapsulating siRNA condensed by poly-L-lysine or stearyl-Arg8. It was known that protamine is an excellent DNA condensing agent.[97] Qin *et al*. developed also a Tat-Cholesterol-modified liposome in which this CPP is covalently bound to cholesterol as hydrophobic anchor. The biological properties of Tat peptide and the stability conferred by the cholesterol allowed Tat-Chol-modified liposomes to cross the BBB.[98]

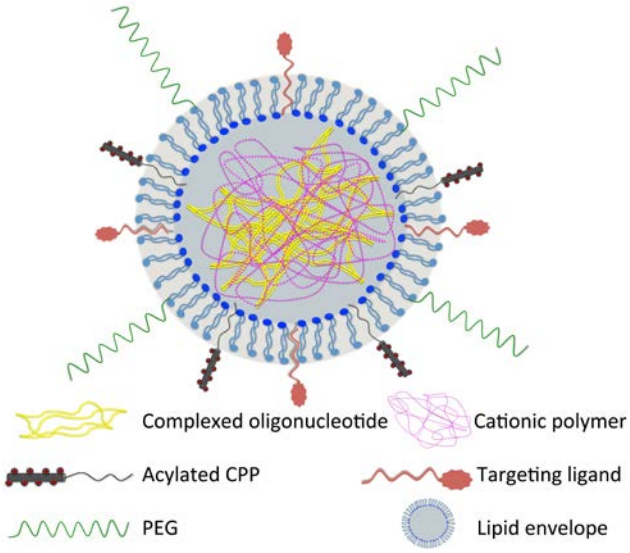


Figure 5: Multifunctional envelope-type nanodevice (MEND) as a functional platform for the intracellular delivery of nucleic acid. Acylated cell-penetrating peptides are exclusively represented on the outer leaflet of the vesicles because stearylated oligoarginines are not prone to spontaneously translocate through membrane bilayers (see section 2.2). Nevertheless, the amount of translocated lipopeptide has never been experimentally evaluated and it cannot be ruled out that some of them might be located on the inner leaflet of the vesicles.

4.2. Targeted multifunctional envelope-type nanodevices

Nanoparticles also passively target the encapsulated molecules to pathological tissues. To increase the selectivity, dual-ligand based PEG-liposomes were designed.

Kribia *et al*. proposed to decorate MENDs with multiple labels: stearyl-Arg8, PEG and an RGD peptide (RGD peptides non-covalently associated to 2000-carbamyl distearoylphosphatidyl-ethanolamine). RGD is a specific ligand for integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ that are highly expressed on the surface of different cancer cells.[99] The uptake of such MEND dual-ligand, stearyl-Arg8/RGD, PEG-Liposome mainly proceeds *via* the clathrin-mediated endocytotic pathway in HUVEC cells. In the presence of only the RGD sequence the MEND is mainly taken up by the caveolae-mediated endocytosis. On the contrary, when only the CPP stearyl-Arg8 decorates the surface, this MEND is taken up by clathrin-mediated endocytosis.[100]

Very recently, Liu *et al*. have reported the use of a DOPE-Tat(AAN) based liposome (~100 nm), in which the Tat(AAN) peptide is anchored to the membrane *via* a DOPE PL. Tat(AAN) is a pro-CPP containing a legumain cleavage site. Legumain is an enzyme over-expressed on the surface of tumor cells. Upon removal of the Ala-Ala-Asn caging tripeptide, the fully functional Tat peptide still bound to DOPE is unmasked allowing a specific internalization of the liposome and thus of the corresponding cargo.[101]

4.3. The mechanisms of internalization and endosomal escape of liposomes are crucial issues

The endosomal escape is a crucial issue and can be induced by the presence of pH-sensitive lipids in the lipid envelope, while the nuclear translocation can be achieved by attaching a nuclear localization system (NLS) to the encapsulated pDNA.

Akita *et al*. set up a method to quantitatively analyze the intracellular distribution of rhodamine-labeled pDNA and the consequent efficacy of the transgene expression with sequential Z-series images captured by confocal microscopy. They showed that a MEND encapsulating pDNA decorated with stearyl-Arg8 on the surface exhibits endosomal escape and nuclear translocation, but lower than LipofectAMINE®. The efficacy of LipofectAMINE® transfection is presumably related to a rapid endosomal escape (cytoplasmic pDNA levels are severely decreased at 4 °C).[102] Later on, the same authors have described how the stearyl-Arg8 modification of liposomes and MENDs can influence the cellular uptake pathways. They report a shift of the uptake mechanism from a clathrin-mediated endocytosis, in the case of low stearyl-Arg8 density, to a macropinocytosis-mediated uptake of liposomes with a high stearyl-Arg8 density. [103] The macropinocytosis-mediated uptake provides a reduced lysosomal degradation hence influencing the intracellular trafficking and resulting in an enhanced gene expression.[103,104]

El-Sayed *et al.*, investigated the role of cell-penetrating sequences decorating the surface of liposomes with respect to the cytosolic release pathway. In fact, high-density octaarginine- and octalysine-modified liposomes are both taken up primarily *via* macropinocytosis but then have different modes of escape from endosomes. Stearyl-Arg8 stimulates efficient escape from endocytotic vesicles *via* a fusion mechanism between the liposomes and the endosomal membrane that works at both neutral and acidic pH. On the contrary, stearyl-Lys8 mediates the escape only at neutral pH. As a consequence, stearyl-Arg8 liposomes display an enhanced gene expression than Lys8-liposomes.[105] The mechanism of the cytoplasmic transport of stearyl-Arg8-liposomes seems to be determined by a microtubule-dependent transport.[106]

To resume, properly decorated nanoparticles can be designed and applied for a successful targeted cancer therapy. All findings herein reported highlight how the cytosolic trafficking events are a crucial issue and, once dissected, can be used to tune the intracellular targeting.

Summary and Conclusion

The addition of a hydrophobic moiety to the sequence of polycationic CPPs dramatically expands the scope of their application as drug delivery agent. Whereas CPPs are usually used as covalent (single molecule) vectors or as non-covalent poorly defined CPP-cargo complexes, acylated CPPs can be conveniently used to create or decorate well-characterized supramolecular nanocarriers. Moreover, the variety of cargoes (small molecules, peptides, nucleic acid or proteins) that were delivered using acylated CPPs demonstrates the versatility of this strategy.

For *in vitro* applications, cationic CPPs can be conveniently used in combination with PyB to enhance the delivery efficiency of the cargo. Reversible complexation of the CPP by PyB temporarily reverses the polarity of the polycationic CPP and allows its transitory solubilization in the membrane bilayer. The CPP and its cargo can ultimately be released inside the cytosol. This strategy presents three important advantages: the cell population internalizes more homogeneously the cargo, higher intracellular concentration of the cargo might be reached and the direct translocation pathway is specifically enhanced. Thus, a co-incubation in the presence of PyB as counteranion might result in an increased accessibility of the cargo to the cytosol. The high flexibility of this strategy makes it very suitable for the optimization of an *in vitro* delivery system based on cationic CPPs. Nevertheless, the formation of a weak ion pair between

cationic residues and hydrophobic counteranions is required. This induces a high sensitivity to anionic competitors and the incubation has to proceeds in PBS buffer, representing an insurmountable barrier for further *in vivo* development.

Permanent change in the amphiphilic character of CPP can be induced by the covalent introduction of a hydrophobic chain. Contrary to the addition of hydrophobic counteranion, the covalent addition of a hydrophobic group to a polyarginine CPP dramatically and irreversibly changes its amphiphaticity: the non-amphipathic Arg9 or Tat peptides become primary amphipathic lipopeptides. The acylation of TP10 increases its primary amphipathic character. As a consequence, CPPs derivatized by hydrocarbons have a high propensity to aggregate in aqueous media. The supramolecular architecture of these aggregates can be finely tuned by changing the nature and the number of fatty acid chains. Interestingly these micelles have the CPP moieties exposed on the surface and a hydrophobic core that can be used to encapsulate hydrophobic drugs to be delivered. Anionic nucleic acids can be also delivered by non-covalent strategy. In this case, the aggregates formed simply by mixing the oligonucleotide with the lipopeptide are large and have never been fully characterized. The acylation of the CPP enhances the stability of the CPP-oligonucleotide complex and its internalization. In such case the internalization occurs *via* an endocytic route and escape from lysosomes has been observed. Alternatively, if the lipopeptide remains monomeric in solution or if the aggregate is sufficiently labile to dissociate upon binding to the outer leaflet of the plasma membrane the acylated CPP can reach the cytosol *via* direct translocation. These general observations might help to design more efficient vectors depending on the most favorable pathway for the considered cargo.

Despite cell-penetrating lipopeptides are still not in commercial use, some of them can be already envisioned as substitutes of the LipofectAMINE® reagent for the delivery *in vitro* delivery of oligonucleotides and might even increase the transfection efficiencies.[51,74,75]

Because of their primary amphipathic structure, acylated cationic CPPs have been also used to decorate the surface of various objects such as vesicular nanocarriers. The CPP moiety itself is supposed to actively enhance the internalization of the vesicle platform *via* energy-dependent processes. Even if the role of acyl chain might be far more complex, for example during an eventual fusion with the endosomal membrane, it is always considered as a simple anchor to the membrane. Vesicular nanocarriers have the advantage to have a

highly tunable surface and an active targeting units can be added to enhance the specificity and thus to compensate the major drawback of CPPs. Because the targeting unit and the CPP are distinct on the surface, both can be *a priori* independently optimized. To date, MENDs are the most integrated and versatile delivery system.

Similarly to CPP, a fine rationalization of the internalization properties of cationic lipopeptides is still lacking. In particular, biophysical experiments are now required to characterize the aggregation and the internalization properties of LPA in order to optimize their delivery properties or even to propose a rational design. Thus, the minimization of the chemical structure of LPA might be an attractive objective. Indeed, the pharmacomodulation of minimalist lipopeptides could be quicker to perform and their in-solution synthesis might be more cost-efficient. The peptidic moiety can be reduced down to 3-4 amino acids, even if such small sequences are themselves non-penetrating in the absence of a hydrophobic moiety. Issues in this research route will be the reduced solubility of the minimalist lipopeptides and their reduced interaction with the cell membrane.

Acknowledgements

The authors are grateful to Prof. Solange Lavielle for fruitful discussion and for her abundant constructive criticism.

Funding

This work was supported in part by the ANR # J12R139 « ELIPTIC ».

References

1 Reymond J-L, Awale M. Exploring Chemical Space for Drug Discovery Using the Chemical Universe Database. *ACS Chem. Neurosci.* 2012; **3**: 649–657; DOI:10.1021/cn3000422.
2 Garcia-Serna R, Mestres J. Anticipating Drug Side Effects by Comparative Pharmacology. *Expert Opin. Drug Metab. Toxicol.* 2010; **6**: 1253–1263; DOI:10.1517/17425255.2010.509343.
3 Munos B. Lessons from 60 Years of Pharmaceutical Innovation. *Nat. Rev. Drug Discov.* 2009; **8**: 959–968; DOI:10.1038/nrd2961.
4 Castanotto D, Rossi JJ. The Promises and Pitfalls of RNA-Interference-Based Therapeutics. *Nature* 2009; **457**: 426–433; DOI:10.1038/nature07758.
5 Ferber D. Gene Therapy. Safer and Virus-Free? *Science* 2001; **294**: 1638–1642; DOI:10.1126/science.294.5547.1638.
6 Dalby B. Advanced Transfection with Lipofectamine 2000 Reagent: Primary Neurons, siRNA, and High-Throughput Applications. *Methods* 2004; **33**: 95–103; DOI:10.1016/j.ymeth.2003.11.023.
7 Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A. Cell Internalization of the Third Helix of the Antennapedia Homeodomain Is Receptor-Independent. *J. Biol. Chem.* 1996; **271**: 18188–18193.
8 Swiecicki J-M, Bartsch A, Tailhades J, Di Pisa M, Heller B, Chassaing G, Mansuy C, Burlina F,

Lavielle S. The Efficacies of Cell-Penetrating Peptides in Accumulating in Large Unilamellar Vesicles Depend on Their Ability To Form Inverted Micelles. *ChemBioChem* 2014; **15**: 884–891; DOI:10.1002/cbic.201300742.
9 Derossi D, Joliot AH, Chassaing G, Prochiantz A. The Third Helix of the Antennapedia Homeodomain Translocates through Biological Membranes. *J. Biol. Chem.* 1994; **269**: 10444.
10 Vivès E, Brodin P, Lebleu B. A Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates through the Plasma Membrane and Accumulates in the Cell Nucleus. *J. Biol. Chem.* 1997; **272**: 16010–16017.
11 Koren E, Torchilin VP. Cell-Penetrating Peptides: Breaking through to the Other Side. *Trends Mol. Med.* 2012; **18**: 385–393; DOI:10.1016/j.molmed.2012.04.012.
12 Jiao C-Y, Delaroche D, Burlina F, Alves ID, Chassaing G, Sagan S. Translocation and Endocytosis for Cell-Penetrating Peptide Internalization. *J. Biol. Chem.* 2009; **284**: 33957–33965; DOI:10.1074/jbc.M109.056309.
13 Rothbard J, Jessop T, Wender P. Adaptive Translocation: The Role of Hydrogen Bonding and Membrane Potential in the Uptake of Guanidinium-Rich Transporters into Cells. *Adv. Drug Deliv. Rev.* 2005; **57**: 495–504; DOI:10.1016/j.addr.2004.10.003.
14 Di Pisa M, Chassaing G, Swiecicki J-M. Translocation Mechanism(s) of Cell-Penetrating Peptides: Biophysical Studies Using Artificial Membrane Bilayers. *Biochemistry (Mosc.)* 2014; DOI:10.1021/bi501392n.
15 Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The Design, Synthesis, and Evaluation of Molecules That Enable or Enhance Cellular Uptake: Peptoid Molecular Transporters. *Proc. Natl. Acad. Sci.* 2000; **97**: 13003–13008; DOI:10.1073/pnas.97.24.13003.
16 Soomets U, Lindgren M, Gallet X, Hällbrink M, Elmquist A, Balaspiri L, Zorko M, Pooga M, Brasseur R, Langel U. Deletion Analogues of Transportan. *Biochim. Biophys. Acta* 2000; **1467**: 165–176.
17 Pooga M, Hällbrink M, Zorko M, Langel U. Cell Penetration by Transportan. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 1998; **12**: 67–77.
18 Reissmann S. Cell Penetration: Scope and Limitations by the Application of Cell-Penetrating Peptides. *J. Pept. Sci.* 2014; **20**: 760–784; DOI:10.1002/psc.2672.
19 Copolovici DM, Langel K, Eriste E, Langel Ü. Cell-Penetrating Peptides: Design, Synthesis, and Applications. *ACS Nano* 2014; **8**: 1972–1994; DOI:10.1021/nn4057269.
20 Brasseur R, Divita G. Happy Birthday Cell Penetrating Peptides: Already 20 Years. *Biochim. Biophys. Acta BBA - Biomembr.* 2010; **1798**: 2177–2181; DOI:10.1016/j.bbamem.2010.09.001.
21 Stanzl EG, Trantow BM, Vargas JR, Wender PA. Fifteen Years of Cell-Penetrating, Guanidinium-Rich Molecular Transporters: Basic Science, Research Tools, and Clinical Applications. *Acc. Chem. Res.* 2013; **46**: 2944–2954; DOI:10.1021/ar4000554.
22 Bechara C, Sagan S. Cell-Penetrating Peptides: 20years Later, Where Do We Stand? *FEBS Lett.* 2013; **587**: 1693–1702; DOI:10.1016/j.febslet.2013.04.031.
23 Almeida PF. Membrane-Active Peptides: Binding, Translocation, and Flux in Lipid Vesicles. *Biochim. Biophys. Acta* 2014; **1838**: 2216–2227; DOI:10.1016/j.bbamem.2014.04.014.
24 Brock R. The Uptake of Arginine-Rich Cell-Penetrating Peptides: Putting the Puzzle Together. *Bioconjug. Chem.* 2014; DOI:10.1021/bc500017t.
25 Shi N-Q, Qi X-R, Xiang B, Zhang Y. A Survey on "Trojan Horse" Peptides: Opportunities, Issues and Controlled Entry to "Troy." *J. Control. Release Off. J. Control. Release Soc.* 2014; **194C**: 53–70; DOI:10.1016/j.jconrel.2014.08.014.
26 Huang Y, Jiang Y, Wang H, Wang J, Shin MC, Byun Y, He H, Liang Y, Yang VC. Curb Challenges of the "Trojan Horse" Approach: Smart Strategies in Achieving Effective yet Safe Cell-Penetrating Peptide-Based Drug Delivery. *Adv. Drug Deliv. Rev.* 2013; **65**: 1299–1315; DOI:10.1016/j.addr.2012.11.007.
27 Ziegler A. Thermodynamic Studies and Binding Mechanisms of Cell-Penetrating Peptides with Lipids and Glycosaminoglycans. *Adv. Drug Deliv. Rev.* 2008; **60**: 580–597; DOI:10.1016/j.addr.2007.10.005.
28 Nishihara M, Perret F, Takeuchi T, Futaki S, Lazar AN, Coleman AW, Sakai N, Matile S. Arginine

Magic with New Counterions up the Sleeve. *Org. Biomol. Chem.* 2005; **3**: 1659–1669; DOI:10.1039/B501472G.
29 Perret F, Nishihara M, Takeuchi T, Futaki S, Lazar AN, Coleman AW, Sakai N, Matile S. Anionic Fullerenes, Calixarenes, Coronenes, and Pyrenes as Activators of Oligo/Polyarginines in Model Membranes and Live Cells. *J. Am. Chem. Soc.* 2005; **127**: 1114–1115; DOI:10.1021/ja043633c.
30 Takeuchi T, Kosuge M, Tadokoro A, Sugiura Y, Nishi M, Kawata M, Sakai N, Matile S, Futaki S. Direct and Rapid Cytosolic Delivery Using Cell-Penetrating Peptides Mediated by Pyrenebutyrate. *ACS Chem. Biol.* 2006; **1**: 299–303; DOI:10.1021/cb600127m.
31 Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. A Comprehensive Model for the Cellular Uptake of Cationic Cell-Penetrating Peptides. *Traffic* 2007; **8**: 848–866.
32 Guterstam P, Madani F, Hirose H, Takeuchi T, Futaki S, EL Andaloussi S, Gräslund A, Langel Ü. Elucidating Cell-Penetrating Peptide Mechanisms of Action for Membrane Interaction, Cellular Uptake, and Translocation Utilizing the Hydrophobic Counter-Anion Pyrenebutyrate. *Biochim. Biophys. Acta BBA - Biomembr.* 2009; **1788**: 2509–2517; DOI:10.1016/j.bbamem.2009.09.014.
33 Sakai N, Matile S. Anion-Mediated Transfer of Polyarginine across Liquid and Bilayer Membranes. *J Am Chem Soc* 2003; **125**: 14348–14356; DOI:10.1021/ja037601l.
34 Inomata K, Ohno A, Tochio H, Isogai S, Tenno T, Nakase I, Takeuchi T, Futaki S, Ito Y, Hiroaki H, Shirakawa M. High-Resolution Multi-Dimensional NMR Spectroscopy of Proteins in Human Cells. *Nature* 2009; **458**: 106–109; DOI:10.1038/nature07839.
35 Katayama S, Nakase I, Yano Y, Murayama T, Nakata Y, Matsuzaki K, Futaki S. Effects of Pyrenebutyrate on the Translocation of Arginine-Rich Cell-Penetrating Peptides through Artificial Membranes: Recruiting Peptides to the Membranes, Dissipating Liquid-Ordered Phases, and Inducing Curvature. *Biochim. Biophys. Acta BBA - Biomembr.* 2013; **1828**: 2134–2142; DOI:10.1016/j.bbamem.2013.05.016.
36 Sakai N, Takeuchi T, Futaki S, Matile S. Direct Observation of Anion-Mediated Translocation of Fluorescent Oligoarginine Carriers into and across Bulk Liquid and Anionic Bilayer Membranes. *ChemBioChem* 2005; **6**: 114–122; DOI:10.1002/cbic.200400256.
37 Schultz AM, Henderson LE, Oroszlan S. Fatty Acylation of Proteins. *Annu. Rev. Cell Biol.* 1988; **4**: 611–647; DOI:10.1146/annurev.cb.04.110188.003143.
38 Resh MD. Trafficking and Signaling by Fatty-Acylated and Prenylated Proteins. *Nat. Chem. Biol.* 2006; **2**: 584–590; DOI:10.1038/nchembio834.
39 Peisajovich SG, Gallo SA, Blumenthal R, Shai Y. C-Terminal Octylation Rescues an Inactive T20 Mutant: Implications for the Mechanism of HIV/SIMIAN Immunodeficiency Virus-Induced Membrane Fusion. *J. Biol. Chem.* 2003; **278**: 21012–21017; DOI:10.1074/jbc.M212773200.
40 Ingallinella P, Bianchi E, Ladwa NA, Wang Y-J, Hrin R, Veneziano M, Bonelli F, Ketas TJ, Moore JP, Miller MD, Pessi A. Addition of a Cholesterol Group to an HIV-1 Peptide Fusion Inhibitor Dramatically Increases Its Antiviral Potency. *Proc. Natl. Acad. Sci.* 2009; **106**: 5801–5806; DOI:10.1073/pnas.0901007106.
41 O'Brian CA, Ward NE, Liskamp RM, de Bont DB, Earnest LE, van Boom JH, Fan D. A Novel N-Myristylated Synthetic Octapeptide Inhibits Protein Kinase C Activity and Partially Reverses Murine Fibrosarcoma Cell Resistance to Adriamycin. *Invest. New Drugs* 1991; **9**: 169–179.
42 Eichholtz T, de Bont DB, de Widt J, Liskamp RM, Ploegh HL. A Myristoylated Pseudosubstrate Peptide, a Novel Protein Kinase C Inhibitor. *J. Biol. Chem.* 1993; **268**: 1982–1986.
43 Ekrami HM, Kennedy AR, Shen WC. Water-Soluble Fatty Acid Derivatives as Acylating Agents for Reversible Lipidization of Polypeptides. *FEBS Lett.* 1995; **371**: 283–286.
44 Vijayaraghavan S, Goueli SA, Davey MP, Carr DW. Protein Kinase A-Anchoring Inhibitor Peptides Arrest Mammalian Sperm Motility. *J. Biol. Chem.* 1997; **272**: 4747–4752; DOI:10.1074/jbc.272.8.4747.
45 Legendre JY, Trzeciak A, Bohrmann B, Deuschle U, Kitas E, Supersaxo A. Dioleoylmelittin as a Novel Serum-Insensitive Reagent for Efficient Transfection of Mammalian Cells. *Bioconjug. Chem.* 1997; **8**: 57–63; DOI:10.1021/bc960076d.
46 Niidome T, Urakawa M, Takaji K, Matsuo Y, Ohmori N, Wada A, Hirayama T, Aoyagi H. Influence of Lipophilic Groups in Cationic Alpha-Helical Peptides on Their Abilities to Bind with DNA and

Deliver Genes into Cells. *J. Pept. Res.* 1999; **54**: 361–367.
47 Carrigan CN, Imperiali B. The Engineering of Membrane-Permeable Peptides. *Anal. Biochem.* 2005; **341**: 290–298; DOI:10.1016/j.ab.2005.03.026.
48 Lee JS, Tung C-H. Lipo-Oligoarginines as Effective Delivery Vectors to Promote Cellular Uptake. *Mol. Biosyst.* 2010; **6**: 2049; DOI:10.1039/c004684a.
49 Peitzsch RM, McLaughlin S. Binding of Acylated Peptides and Fatty Acids to Phospholipid Vesicles: Pertinence to Myristoylated Proteins. *Biochemistry (Mosc.)* 1993; **32**: 10436–10443.
50 Silvius JR, l' Heureux F. Fluorometric Evaluation of the Affinities of Isoprenylated Peptides for Lipid Bilayers. *Biochemistry (Mosc.)* 1994; **33**: 3014–3022.
51 Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, Harashima H, Sugiura Y. Stearylated Arginine-Rich Peptides: A New Class of Transfection Systems. *Bioconjug. Chem.* 2001; **12**: 1005–1011; DOI:10.1021/bc015508l.
52 Katayama S, Hirose H, Takayama K, Nakase I, Futaki S. Acylation of Octaarginine: Implication to the Use of Intracellular Delivery Vectors. *J. Controlled Release* 2011; **149**: 29–35; DOI:10.1016/j.jconrel.2010.02.004.
53 Mäe M, EL Andaloussi S, Lundin P, Oskolkov N, Johansson HJ, Guterstam P, Langel Ü. A Stearylated CPP for Delivery of Splice Correcting Oligonucleotides Using a Non-Covalent Co-Incubation Strategy. *J. Controlled Release* 2009; **134**: 221–227; DOI:10.1016/j.jconrel.2008.11.025.
54 Pham W, Kircher MF, Weissleder R, Tung C-H. Enhancing Membrane Permeability by Fatty Acylation of Oligoarginine Peptides. *ChemBioChem* 2004; **5**: 1148–1151; DOI:10.1002/cbic.200400063.
55 Pham W, Zhao B-Q, Lo EH, Medarova Z, Rosen B, Moore A. Crossing the Blood–brain Barrier: A Potential Application of Myristoylated Polyarginine for in Vivo Neuroimaging. *NeuroImage* 2005; **28**: 287–292; DOI:10.1016/j.neuroimage.2005.06.007.
56 Langel K, Lindberg S, Copolovici D, Arukuusk P, Sillard R, Langel Ű. Novel Fatty Acid Modifications of Transportan 10. *Int. J. Pept. Res. Ther.* 2010; **16**: 247–255; DOI:10.1007/s10989-010-9224-x.
57 Kim W, Christensen L, Jo S, Yockman J, Jeong J, Kim Y, Kim S. Cholesteryl Oligoarginine Delivering Vascular Endothelial Growth Factor siRNA Effectively Inhibits Tumor Growth in Colon Adenocarcinoma. *Mol. Ther.* 2006; **14**: 343–350; DOI:10.1016/j.ymthe.2006.03.022.
58 Guo XD, Tandiono F, Wiradharma N, Khor D, Tan CG, Khan M, Qian Y, Yang Y-Y. Cationic Micelles Self-Assembled from Cholesterol-Conjugated Oligopeptides as an Efficient Gene Delivery Vector. *Biomaterials* 2008; **29**: 4838–4846; DOI:10.1016/j.biomaterials.2008.07.053.
59 Wollack JW, Zeliadt NA, Mullen DG, Amundson G, Geier S, Falkum S, Wattenberg EV, Barany G, Distefano MD. Multifunctional Prenylated Peptides for Live Cell Analysis. *J. Am. Chem. Soc.* 2009; **131**: 7293–7303; DOI:10.1021/ja805174z.
60 Khalil IA, Futaki S, Niwa M, Baba Y, Kaji N, Kamiya H, Harashima H. Mechanism of Improved Gene Transfer by the N-Terminal Stearylation of Octaarginine: Enhanced Cellular Association by Hydrophobic Core Formation. *Gene Ther.* 2004; **11**: 636–644; DOI:10.1038/sj.gt.3302128.
61 Bode SA, Thévenin M, Bechara C, Sagan S, Bregant S, Lavielle S, Chassaing G, Burlina F. Self-Assembling Mini Cell-Penetrating Peptides Enter by Both Direct Translocation and Glycosaminoglycan-Dependent Endocytosis. *Chem. Commun.* 2012; **48**: 7179–7181; DOI:10.1039/C2CC33240J.
62 Kumar M, Medarova Z, Pantazopoulos P, Dai G, Moore A. Novel Membrane-Permeable Contrast Agent for Brain Tumor Detection by MRI. *Magn. Reson. Med.* 2010; **63**: 617–624; DOI:10.1002/mrm.22216.
63 Medarova Z, Pham W, Farrar C, Petkova V, Moore A. In Vivo Imaging of siRNA Delivery and Silencing in Tumors. *Nat. Med.* 2007; **13**: 372–377; DOI:10.1038/nm1486.
64 Dehsorkhi A, Castelletto V, Hamley IW. Self-Assembling Amphiphilic Peptides. *J. Pept. Sci.* 2014; **20**: 453–467; DOI:10.1002/psc.2633.
65 Lim Y, Lee E, Lee M. Controlled Bioactive Nanostructures from Self-Assembly of Peptide Building Blocks. *Angew. Chem. Int. Ed.* 2007; **46**: 9011–9014; DOI:10.1002/anie.200702732.
66 Zhang P, Cheetham AG, Lin Y, Cui H. Self-Assembled Tat Nanofibers as Effective Drug Carrier

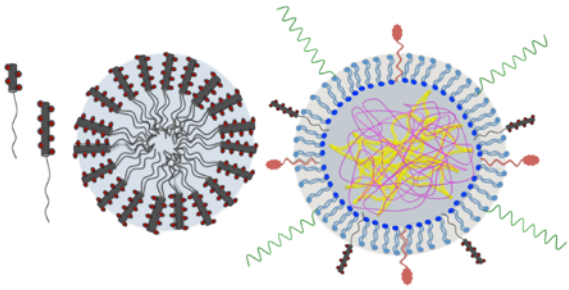
and Transporter. *ACS Nano* 2013; **7**: 5965–5977; DOI:10.1021/nn401667z.
67 Chen J-X, Wang H-Y, Li C, Han K, Zhang X-Z, Zhuo R-X. Construction of Surfactant-like Tetra-Tail Amphiphilic Peptide with RGD Ligand for Encapsulation of Porphyrin for Photodynamic Therapy. *Biomaterials* 2011; **32**: 1678–1684; DOI:10.1016/j.biomaterials.2010.10.047.
68 Kim HH, Lee WS, Yang JM, Shin S. Basic Peptide System for Efficient Delivery of Foreign Genes. *Biochim. Biophys. Acta BBA* 2003; **1640**: 129–136; DOI:10.1016/S0167-4889(03)00028-4.
69 Wadhwa MS, Collard WT, Adami RC, McKenzie DL, Rice KG. Peptide-Mediated Gene Delivery: Influence of Peptide Structure on Gene Expression. *Bioconjug. Chem.* 1997; **8**: 81–88.
70 Emi N, Kidoaki S, Yoshikawa K, Saito H. Gene Transfer Mediated by Polyarginine Requires a Formation of Big Carrier-Complex of DNA Aggregate. *Biochem. Biophys. Res. Commun.* 1997; **231**: 421–424; DOI:10.1006/bbrc.1997.6125.
71 Ifediba MA, Medarova Z, Ng S, Yang J, Moore A. siRNA Delivery to CNS Cells Using a Membrane Translocation Peptide. *Bioconjug. Chem.* 2010; **21**: 803–806; DOI:10.1021/bc900488e.
72 Tönges L, Lingor P, Egle R, Dietz GPH, Fahr A, Bähr M. Stearylated Octaarginine and Artificial Virus-like Particles for Transfection of siRNA into Primary Rat Neurons. *RNA* 2006; **12**: 1431–1438; DOI:10.1261/rna.2252206.
73 Andaloussi SE, Lehto T, Mäger I, Rosenthal-Aizman K, Oprea II, Simonson OE, Sork H, Ezzat K, Copolovici DM, Kurrikoff K, Viola JR, Zaghloul EM, Sillard R, Johansson HJ, Hassane FS, Guterstam P, Suhorutšenko J, Moreno PMD, Oskolkov N, Hälldin J, Tedebark U, Metspalu A, Lebleu B, Lehtiö J, Smith CIE, Langel Ü. Design of a Peptide-Based Vector, PepFect6, for Efficient Delivery of siRNA in Cell Culture and Systemically in Vivo. *Nucleic Acids Res.* 2011; **39**: 3972–3987; DOI:10.1093/nar/gkq1299.
74 Ezzat K, Andaloussi SE, Zaghloul EM, Lehto T, Lindberg S, Moreno PMD, Viola JR, Magdy T, Abdo R, Guterstam P, Sillard R, Hammond SM, Wood MJA, Arzumanov AA, Gait MJ, Smith CIE, Hällbrink M, Langel Ü. PepFect 14, a Novel Cell-Penetrating Peptide for Oligonucleotide Delivery in Solution and as Solid Formulation. *Nucleic Acids Res.* 2011; **39**: 5284–5298; DOI:10.1093/nar/gkr072.
75 Lindberg S, Muñoz-Alarcón A, Helmfors H, Mosqueira D, Gyllborg D, Tudoran O, Langel Ü. PepFect15, a Novel Endosomolytic Cell-Penetrating Peptide for Oligonucleotide Delivery via Scavenger Receptors. *Int. J. Pharm.* 2013; **441**: 242–247; DOI:10.1016/j.ijpharm.2012.11.037.
76 Suhorutsenko J, Oskolkov N, Arukuusk P, Kurrikoff K, Eriste E, Copolovici D-M, Langel Ü. Cell-Penetrating Peptides, PepFects, Show No Evidence of Toxicity and Immunogenicity In Vitro and In Vivo. *Bioconjug. Chem.* 2011; **22**: 2255–2262; DOI:10.1021/bc200293d.
77 Futaki S. Membrane-Permeable Arginine-Rich Peptides and the Translocation Mechanisms. *Adv. Drug Deliv. Rev.* 2005; **57**: 547–558; DOI:10.1016/j.addr.2004.10.009.
78 Aussedat B, Dupont E, Sagan S, Joliot A, Lavielle S, Chassaing G, Burlina F. Modifications in the Chemical Structure of Trojan Carriers: Impact on Cargo Delivery. *Chem. Commun.* 2008; 1398–1400; DOI:10.1039/b800433a.
79 Nasrolahi Shirazi A, Oh D, Tiwari RK, Sullivan B, Gupta A, Bothun GD, Parang K. Peptide Amphiphile Containing Arginine and Fatty Acyl Chains as Molecular Transporters. *Mol. Pharm.* 2013; **10**: 4717–4727; DOI:10.1021/mp400539r.
80 Oh D, Nasrolahi Shirazi A, Northup K, Sullivan B, Tiwari RK, Bisoffi M, Parang K. Enhanced Cellular Uptake of Short Polyarginine Peptides through Fatty Acylation and Cyclization. *Mol. Pharm.* 2014; **11**: 2845–2854; DOI:10.1021/mp500203e.
81 Abes R, Moulton HM, Clair P, Yang S-T, Abes S, Melikov K, Prevot P, Youngblood DS, Iversen PL, Chernomordik LV, Lebleu B. Delivery of Steric Block Morpholino Oligomers by (R-X-R)4 Peptides: Structure–activity Studies. *Nucleic Acids Res.* 2008; **36**: 6343–6354; DOI:10.1093/nar/gkn541.
82 Fujita T, Furuhata M, Hattori Y, Kawakami H, Toma K, Maitani Y. High Gene Delivery in Tumor by Intratumoral Injection of Tetraarginine-PEG Lipid-Coated protamine/DNA. *J. Controlled Release* 2008; **129**: 124–127; DOI:10.1016/j.jconrel.2008.04.010.
83 Obata Y, Suzuki D, Takeoka S. Evaluation of Cationic Assemblies Constructed with Amino Acid Based Lipids for Plasmid DNA Delivery. *Bioconjug. Chem.* 2008; **19**: 1055–1063; DOI:10.1021/bc700416u.
84 Prata CAH, Zhang X-X, Luo D, McIntosh TJ, Barthelemy P, Grinstaff MW. Lipophilic Peptides for Gene Delivery. *Bioconjug. Chem.* 2008; **19**: 418–420; DOI:10.1021/bc700451b.

85 Gehin C, Montenegro J, Bang E-K, Cajaraville A, Takayama S, Hirose H, Futaki S, Matile S, Riezman H. Dynamic Amphiphile Libraries To Screen for the "Fragrant" Delivery of siRNA into HeLa Cells and Human Primary Fibroblasts. *J. Am. Chem. Soc.* 2013; **135**: 9295–9298; DOI:10.1021/ja404153m.
86 Wender P, Rothbard J, Wright L, Kreider E, VanDeusen C. Transporters Comprising Spaced Arginine Moieties. US20030032593 A1, US20030032593 A1.
87 Lehto T, Abes R, Oskolkov N, Suhorutšenko J, Copolovici D-M, Mäger I, Viola JR, Simonson OE, Ezzat K, Guterstam P. Delivery of Nucleic Acids with a Stearylated (RxR)4 Peptide Using a Non-Covalent Co-Incubation Strategy. *J. Controlled Release* 2010; **141**: 42–51; DOI:10.1016/j.jconrel.2009.08.028.
88 Ye G, Nam N-H, Kumar A, Saleh A, Shenoy DB, Amiji MM, Lin X, Sun G, Parang K. Synthesis and Evaluation of Tripodal Peptide Analogues for Cellular Delivery of Phosphopeptides. *J. Med. Chem.* 2007; **50**: 3604–3617; DOI:10.1021/jm070416o.
89 Ye G, Gupta A, DeLuca R, Parang K, Bothun GD. Bilayer Disruption and Liposome Restructuring by a Homologous Series of Small Arg-Rich Synthetic Peptides. *Colloids Surf. B Biointerfaces* 2010; **76**: 76–81; DOI:10.1016/j.colsurfb.2009.10.016.
90 Gupta A, Mandal D, Ahmadibeni Y, Parang K, Bothun G. Hydrophobicity Drives the Cellular Uptake of Short Cationic Peptide Ligands. *Eur. Biophys. J.* 2011; **40**: 727–736; DOI:10.1007/s00249-011-0685-4.
91 Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic Polyethyleneglycols Effectively Prolong the Circulation Time of Liposomes. *FEBS Lett.* 1990; **268**: 235–237; DOI:10.1016/0014-5793(90)81016-H.
92 Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular Permeability and Interstitial Penetration of Sterically Stabilized (stealth) Liposomes in a Human Tumor Xenograft. *Cancer Res.* 1994; **54**: 3352–3356.
93 Maeda H, Sawa T, Konno T. Mechanism of Tumor-Targeted Delivery of Macromolecular Drugs, Including the EPR Effect in Solid Tumor and Clinical Overview of the Prototype Polymeric Drug SMANCS. *J. Control. Release Off. J. Control. Release Soc.* 2001; **74**: 47–61.
94 Davis ME, Chen Z (Georgia), Shin DM. Nanoparticle Therapeutics: An Emerging Treatment Modality for Cancer. *Nat. Rev. Drug Discov.* 2008; **7**: 771–782; DOI:10.1038/nrd2614.
95 Hatakeyama H, Akita H, Harashima H. A Multifunctional Envelope Type Nano Device (MEND) for Gene Delivery to Tumours Based on the EPR Effect: A Strategy for Overcoming the PEG Dilemma. *Adv. Drug Deliv. Rev.* 2011; **63**: 152–160; DOI:10.1016/j.addr.2010.09.001.
96 Kogure K, Moriguchi R, Sasaki K, Ueno M, Futaki S, Harashima H. Development of a Non-Viral Multifunctional Envelope-Type Nano Device by a Novel Lipid Film Hydration Method. *J. Controlled Release* 2004; **98**: 317–323; DOI:10.1016/j.jconrel.2004.04.024.
97 Moriguchi R, Kogure K, Akita H, Futaki S, Miyagishi M, Taira K, Harashima H. A Multifunctional Envelope-Type Nano Device for Novel Gene Delivery of siRNA Plasmids. *Int. J. Pharm.* 2005; **301**: 277–285; DOI:10.1016/j.ijpharm.2005.05.021.
98 Qin Y, Chen H, Zhang Q, Wang X, Yuan W, Kuai R, Tang J, Zhang L, Zhang Z, Zhang Q, Liu J, He Q. Liposome Formulated with TAT-Modified Cholesterol for Improving Brain Delivery and Therapeutic Efficacy on Brain Glioma in Animals. *Int. J. Pharm.* 2011; **420**: 304–312; DOI:10.1016/j.ijpharm.2011.09.008.
99 Gladson CL, Cheresh DA. Glioblastoma Expression of Vitronectin and the Alpha v Beta 3 Integrin. Adhesion Mechanism for Transformed Glial Cells. *J. Clin. Invest.* 1991; **88**: 1924–1932; DOI:10.1172/JCI115516.
100 Kibria G, Hatakeyama H, Ohga N, Hida K, Harashima H. Dual-Ligand Modification of PEGylated Liposomes Shows Better Cell Selectivity and Efficient Gene Delivery. *J. Controlled Release* 2011; **153**: 141–148; DOI:10.1016/j.jconrel.2011.03.012.
101 Liu Z, Xiong M, Gong J, Zhang Y, Bai N, Luo Y, Li L, Wei Y, Liu Y, Tan X, Xiang R. Legumain Protease-Activated TAT-Liposome Cargo for Targeting Tumours and Their Microenvironment. *Nat. Commun.* 2014; **5**; DOI:10.1038/ncomms5280.
102 Akita H. Quantitative Three-Dimensional Analysis of the Intracellular Trafficking of Plasmid

DNA Transfected by a Nonviral Gene Delivery System Using Confocal Laser Scanning Microscopy. *Mol. Ther.* 2004; **9**: 443–451; DOI:10.1016/j.ymthe.2004.01.005.
103 Khalil IA, Kogure K, Futaki S, Harashima H. High Density of Octaarginine Stimulates Macropinocytosis Leading to Efficient Intracellular Trafficking for Gene Expression. *J. Biol. Chem.* 2006; **281**: 3544–3551; DOI:10.1074/jbc.M503202200.
104 Khalil IA, Kogure K, Futaki S, Hama S, Akita H, Ueno M, Kishida H, Kudoh M, Mishina Y, Kataoka K, Yamada M, Harashima H. Octaarginine-Modified Multifunctional Envelope-Type Nanoparticles for Gene Delivery. *Gene Ther.* 2007; **14**: 682–689; DOI:10.1038/sj.gt.3302910.
105 El-Sayed A, Khalil IA, Kogure K, Futaki S, Harashima H. Octaarginine- and Octalysine-Modified Nanoparticles Have Different Modes of Endosomal Escape. *J. Biol. Chem.* 2008; **283**: 23450–23461; DOI:10.1074/jbc.M709387200.
106 Akita H, Enoto K, Masuda T, Mizuguchi H, Tani T, Harashima H. Particle Tracking of Intracellular Trafficking of Octaarginine-Modified Liposomes: A Comparative Study With Adenovirus. *Mol. Ther.* 2010; **18**: 955–964; DOI:10.1038/mt.2010.33.

For Table of Contents Use Only

The influence of hydrocarbon moieties on the internalization and delivery ability of cell-penetrating peptides is reviewed. The alterations of their physico-chemical characteristics are discussed in order to rationalize some of the emerging properties of cell penetrating lipopeptides. General rules for the design of new lipopeptides are outlined as well as a guideline to choose the appropriate delivery vector.



When cationic cell-penetrating peptides meet hydrocarbons to enhance *in cell* cargo delivery

Margherita Di Pisa*, Gérard Chassaing and Jean-Marie Swiecicki*