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# Effects of cargo molecules on membrane perturbation caused by transportan10 based cell-penetrating peptides

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ABSTRACT

Cell-penetrating peptides with the ability to escape endosomes and reach the target are of great value as delivery vectors for different bioactive cargoes and future treatment of human diseases. We have studied two such peptides, NickFect1 and NickFect51, both originated from stearylated transportan10 (PF3). To obtain more insight into the mechanism(s) of peptide delivery and the biophysical properties of an efficient vector system, we investigated the effect of different bioactive oligonucleotide cargoes on peptide–membrane perturbation and peptide structural induction. We studied the membrane interactions of the peptides with large unilamellar vesicles and compared their effects with parent peptides transportan10 and PF3. In addition, cellular uptake and peptide-mediated oligonucleotide delivery were analyzed. Calcein leakage experiments showed that similar to transportan10, NickFect51 caused a significant degree of membrane leakage, whereas NickFect1, similar to PF3, was less membrane perturbing. The results are in agreement with previously published results indicating that NickFect51 is a more efficient endosomal escaper. However, the presence of a large cargo like plasmid DNA inhibited NickFect's membrane perturbation and cellular uptake efficiency of the peptide was reduced. We conclude that the pathway for cellular uptake of peptide complexes is cargo dependent, whereas the endosomal escape efficacy depends on peptide hydrophobicity and chemical structure. For small interfering RNA delivery, NickFect51 appears to be optimal. The biophysical signature shows that the peptide alone causes membrane perturbation, but the cargo complex does not. These two biophysical characteristics of the peptide and its cargo complex may be the signature of an efficient delivery vector system.



## 1. Introduction

The possibility of selectively and efficiently manipulating eukaryotic gene expression holds much promise for modern medicine. Search has been focused on finding new stable biomolecules and their synthetic analogs that can correct or substitute disease causing genetic information. Among the candidates are nucleic acids, such as double-stranded plasmid DNA (pDNA) [1,2], short single-stranded splice-correcting oligonucleotides (SCO) [3,4] and small interfering RNA (siRNA) [5,6]. Although some recent cases of success can be pointed out [7] the use of these nucleic acid based therapies in clinical applications is limited until present by the poor stability in serum containing media and low uptake into cells due to their high molecular weight, negative charge and hydrophilic nature.

The use of cell-penetrating peptides (CPPs) as delivery vehicles for biomolecular cargoes, such as pDNA, SCOs and siRNAs, offers a set of advantages such as low toxicity and efficiency at reduced doses and some of these peptides are able to promote endosomal escape after cell internalization [8–10]. Generally, CPPs are defined as short and water-soluble peptides, which can be hydrophilic (cationic), hydrophobic or intermediately hydrophobic [11,12]. A major advantage of some CPPs is their capacity of forming stable non-covalent complexes with the cargo [13]. However this strategy often results in entrapment of the CPP/cargo complexes in endosomal vesicles after cellular uptake by endocytosis [14–16]. The necessity to improve endosomal escape has driven the insertion of several chemical modifications to CPPs, such as the addition of trifluoromethylquinoline moieties or replacing certain residues with His to make endosomolytic CPPs [6,17–19].

*Abbreviations:* CPP, cell-penetrating peptide; TP10, transportan10; NickFect, NF; PepFect, PF; FACS, fluorescence activated cell sorting; POPC, palmitoyl-2-oleoyl-phosphatidylcholine; POPG, palmitoyl-2-oleoyl-phosphatidylglycerol; LUV, large unilamellar vesicle; CD, circular dichroism; MR, molar ratio; CR, charge ratio; DLS, dynamic light scattering; SCO, splice-correcting oligonucleotide; pDNA, plasmid DNA; siRNA, small interfering RNA; pGL3, luciferase expressing plasmid; FAM, carboxyfluorescein

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Transportan10 (TP10) is one of the well-known CPPs, a truncated analog of transportan with the N-terminal fragment from the galanin neuropeptide and the C-terminal fragment from the wasp venom peptide mastoparan and an extra lysine positioned between the two fused sequences [20–22]. It belongs to a group of the hydrophobic CPPs with strong affinity to a model membrane already at low concentrations [11]. TP10 showed not only high transduction efficiency but also high cell toxicity [23].

Various TP10 analogs have been developed with the objective to increase the bioavailability, stability and efficiency in cargo delivery. TP10 was modified with stearic acid and gave origin to a new family of stearylated CPPs, named PepFects (PFs). PepFect3 (PF3) is the closest TP10 analog with only an additional stearyl moiety at the N-terminus, allowing the formation of stable peptide/oligonucleotide complexes [4,24]. It has been shown that PF3 has lower affinity for a model membrane compared to unstearylated TP10 [25]. The other family of stearylated TP10 analogs is named NickFects. NickFect1 (NF1) resulted from the replacement of isoleucine8 by a more hydrophilic threonine, and additionally the insertion of a phosphoryl group to tyrosine3 in the stearylated TP10 (PF3) sequence. These modifications increase its hydrophilicity and reduce its charge leading to a pH-dependent peptide vector with a good endosomolytic capacity [26–28]. NickFect51 (NF51) has a kink resulted from two simultaneous modifications to PF3, namely the replacement of lysine7 with ornithine and the use of δ-$NH_2$ group of ornithine7 for subsequent synthesis instead of α-$NH_2$ (Table 1). This modification enhances the stability of the complexes in the cytosol and their endosomal escape [29–31]. It has been also found that NF51 functions as an efficient CPP in protein production system [27].

The number of CPPs is increasing including both protein-derived and designed peptides with different physico-chemical properties [32]. However, there is still space for improvement regarding the efficacy of CPPs in the presence of serum as well as to enhance their endosomal escape and biological activities [33]. In addition, both the cellular internalization and endosomal escape mechanisms of CPPs are not well understood. Understanding the molecular mechanisms underlying the CPP cellular uptake and membrane translocation is a necessary prerequisite to characterize the structural basis for modulation of these peptides. Although several parameters may simultaneously affect their biological responses, phospholipid membrane interaction plays a major role in their cellular uptake and endosomal escape efficiencies and hence their biological activities.

It should be emphasized that the presence of cargo can alter the mechanism of internalization as well as endosomal escape efficiency. Cargo characteristics such as size, charge and conjugation methodology have been shown to influence the CPP translocation mechanism [17,34–38]. However there are few studies concerning the effect of cargo on CPP–membrane interaction and perturbation. One of these studies showed that streptavidin protein covalently bound to TP10 significantly decreases the amount of leakage caused by the peptide while smaller cargo has no obvious effect on peptide–membrane perturbation [39]. In 2002, Fischer et al. [37] evaluated CPPs for the controlled import of small molecules. They studied the dependence of the CPP driven import efficiency for different conjugated fluorophores and for the nature of cargo. They have reported an independence of the nature of the fluorophore contrasted by a marked dependence of the peptide cargo [37]. More recently, Freire et al. [40] developed a novel mathematical lipid partition model, which allows estimating lipid–water partition constants of supramolecular CPP–cargo complexes from fluorescence spectroscopy data. In their work they tested the partition extent of two membrane active peptides derived from dengue virus capsid protein (DENV C protein) with potential CPP properties, both free and in the presence of ssDNA molecular cargo. According to the authors, deducing carrier properties from studies using free CPPs is limited, due to the structural and chemical rearrangements revealed when part of supramolecular complexes [40].

This work aims to investigate the membrane bilayer interaction of three different TP10 analogs, PF3, NF1 and NF51, in the presence or absence of a cargo. These TP10 modified peptides have shown different biological activities when they are non-covalently attached to cargo molecules [4,26,27]. An obvious question is what variables could drive the membrane interaction of CPP and CPP/cargo complexes, enhance the membrane perturbation and therefore make them more efficient delivery vectors.

We employed large unilamellar vesicles (LUVs) as a bio-membrane model system to study the abovementioned peptides. LUVs are a simplified and relevant membrane mimetic system for studying peptide–membrane interaction and peptide structure induction in the presence of the membrane. Both neutral vesicles composed of zwitterionic POPC and partially negatively charged vesicles composed of POPC/POPG (7:3) phospholipids were used. Three different spectroscopic methods were used including fluorescence spectroscopy to investigate peptide induced membrane leakage, circular dichroism (CD) spectroscopy to evaluate secondary structure induction and dynamic light scattering (DLS) to measure the evolution of size. Surface charge using zeta-potential was also measured for the pure peptide and peptide/cargo complexes in water solution. Besides biophysical techniques, we studied the impact of the cargo on the peptide ability to gain intracellular access utilizing fluorescence activated cell sorting (FACS) on live cells. In addition, functional assays were used to evaluate and compare their potentials in delivery of biomolecules. Altogether, the results may

**Table 1**
Peptides and the respective cargo complexes investigated in this work together with their physico-chemical properties.

| CPP | PF3 | NF1 | NF51 |
|---|---|---|---|
| Sequence[a] | Stearyl-AGYLLGKINLKALAALAKKIL[b] | Stearyl-AGY($PO_3$)LLGKTNLKALAALAKKIL[b] | Stearyl-AGYLLG)δ-OINLKALAALAKKIL[b] |
| Average hydrophobicity[c] | 0.95 | 0.35 | 0.99 |
| Positive charge | 4 | 3 | 4 |
| Concentration (μM)[d] | 5 | 8 | 2 |
| CPP/pGL3 | | | |
| CR[e] | (3:1) | (3:1) | (3:1) |
| MR | (7200:1) | (7200:1) | (7200:1) |
| CPP/SCO | | | |
| CR | (2:1) | (1.5:1) | (2:1) |
| MR | (10:1) | (10:1) | (10:1) |
| CPP/siRNA | | | |
| CR | (2:1) | (1.5:1) | (2:1) |
| MR | (20:1) | (20:1) | (20:1) |

[a] All peptides are N-terminally stearylated (stearyl stands for $CH_3(CH_2)_{16}CO-$).
[b] All peptides have amidated C-terminus and peptides used for cellular uptake experiments were labeled with FAM to ε-$NH_2$ group of additional lysine at the C-terminus.
[c] Average amino acid hydrophobicity was calculated according to [40].
[d] Based on the peptide potency in leakage induction, different peptide concentrations were used for peptide/cargo complex preparation in the leakage and CD experiments.
[e] CR and MR refer to charge ratio and molar ratio, respectively.

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