Expert Opinion

Chemically modified cell-penetrating peptides for the delivery of nucleic acids

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Short nucleic acids targeting biologically important RNAs and plasmids have been shown to be promising future therapeutics; however, their hydrophilic nature greatly limits their utility in clinics and therefore efficient delivery vectors are greatly needed. Cell-penetrating peptides (CPPs) are relatively short amphipathic and/or cationic peptides that are able to transport various biologically active molecules inside mammalian cells, both *in vitro* and *in vivo*, in a seemingly non-toxic fashion. Although CPPs have proved to be appealing drug delivery vehicles, their major limitation in nucleic acid delivery is that most of the internalized peptide-cargo is entrapped in endosomal compartments following endocytosis and the bioavailability is therefore severely reduced. Several groups are working towards overcoming this obstacle and this review highlights the evidence that by introducing chemical modification in CPPs, the bioavailability of delivered nucleic acids increases significantly.

Keywords: chemical modifications, endosomal escape, gene therapy, non-covalent co-incubation

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1. Introduction

The discovery of mutations in genes causing serious human diseases has increased the demand for efficient and safe delivery vectors for plasmids and oligonucleotides (ONs) for the purpose of gene therapy. Generally, gene therapy aims to treat or alleviate diseases such as inherited disorders or cancers by genetically modifying the cells of interest. It can be divided broadly into two subgroups: i) the mutated or missing gene is replaced in targeted cells with a correct gene using donor DNA in order to obtain a fully functional gene product; and ii) the targeted gene expression is inhibited or genetic defect is corrected and normal gene expression is restored with antisense ONs. Most viral and non-viral delivery vectors for nucleic acids available so far have been formulated and optimized for the delivery of gene-expressing plasmids. Although viral vectors fulfill all criteria for efficient transport, including adsorption to the cell surface, uptake by the cell, endosomal escape, nuclear translocation and expression of the gene, they potentially suffer from several detrimental effects, such as acute immune responses, viral recombination and unwanted random chromosomal integration. Furthermore, limitations in DNA carrying capacity and issues related to the production of viral vectors create further practical challenges. More importantly, they are not compatible with the transient delivery of short ONs. Therefore, significant efforts have been made to develop safe and efficient non-viral vectors. The most commonly used non-viral vehicles are cationic liposomes and polycations that in addition to plasmids are applicable for the delivery of short ONs. However, their *in vivo* efficacy is generally low and they are limited owing to acute immune responses and toxicity to cells, as reviewed in [1].

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A class of peptides, cell-penetrating peptides (CPPs), also termed protein transduction domains (PTDs), has recently gained increasing attention as efficient non-viral delivery vectors. CPPs are mainly short amphipathic and/or cationic peptides capable of transporting various hydrophilic molecules over the plasma membrane. Since the discovery of the first CPPs, including penetratin [2], Tat(48-60) [3], transportan (TP) [4] and oligoarginine [5], hundreds of new CPPs have emerged. Although the uptake mechanism of CPPs is still debated, new findings in the research field during the past few years have contributed to the understanding of how these short peptides make their way into the cell interior. It is important to gain information regarding their uptake mechanism in order to be able to rationally design new CPPs or to modify existing ones to improve cargo transport into specific cellular compartments. Based on several investigations, four main events for CPP uptake have been suggested: first, interactions with the cell surface; second, translocation through the cell membrane mainly through different types of endocytosis, or direct translocation through the cell membrane has also been reported [6-8]; third, destination to different intracellular compartments; and fourth, in the case of endocytic uptake, endosomal release of the CPPs. Independent of the subsequent cellular uptake route, it is believed that internalization of CPPs or CPP–cargo complexes begins with interactions with components on the surface of the plasma membrane. It has been shown that this occurs through binding to cell surface proteoglycans, and in particular heparane sulfate proteoglycans [9-14]. Also, hydrophobic interactions between CPP sequences and cell membrane lipids have been proposed to be important in creating the first contact [12]. As mentioned above, most CPPs are believed to utilize mainly endocytosis to translocate over the plasma membrane, which raises a major concern – entrapment of the delivered cargos in endosomal compartments that result in limited activity of the cargo. This issue is unquestionably one of the biggest weaknesses of not only CPPs but also most other transfection systems for ONs, and is therefore being discussed in more detail later on. First, a brief background is given on the different classes of ONs that are exploited to interfere with gene expression.

2. Antisense technology

There is a growing repertoire of antisense mechanisms that can be exploited for post-transcriptional gene silencing. Classical antisense technology makes use of short single-stranded antisense ONs that interact with target mRNA in a sequence-specific manner and causes translational arrest of mRNA either through the recruitment of RNase H that degrades the target sequence or, less commonly, via sterical hindrance, that is, preventing ribosomal assembly on mRNA [15]. The latter antisense mechanism has recently gained increasing attention for targeting of pre-mRNAs in order to manipulate splicing patterns and to correct aberrant splicing in various diseases. This so-called splice correction or splice switching platform makes use of RNase H-incompetent ONs in order to avoid degradation of pre-mRNAs [16]. Similarly, single-stranded ONs have recently been used extensively for silencing of regulatory micro-RNAs, that is, the anti-miR approach, which utilizes the same principle as classical antisense but instead targets complementary micro-RNAs rather than mRNAs [17]. Another ON-based strategy that has been used to interfere with gene regulation is the decoy approach. This method is designed to alter the activity of transcription factors. As transcription factors can recognize relatively short binding sequences, double-stranded decoy ONs bearing consensus binding sites can be used as a means of sequestering transcription factors by competing with the genome for binding [18]. However, the most recognized gene silencing approach is RNA interference, first described by Fire *et al.*, where double-stranded short interfering RNA (siRNA) duplexes are used to silence gene expression by means of degradation of mRNA [19].

CPPs have readily been conjugated to the above-mentioned ONs, usually by means of a disulfide bridge that is cleaved when exposed to a reductive environment (i.e., the cytoplasm), thus releasing the ON from CPP. An increasing number of studies have recently substantiated the utility of CPPs for the tranfection of siRNAs, decoy ONs, and splice correcting ONs (SCOs). A selection of CPPs and delivered ON-cargos are listed in **Table 1**, and some examples are described in the next section.

2.1 Delivery of oligonucleotides using cell-penetrating peptides

Since the antennapedia protein was shown to effectively deliver antisense ONs targeting the amyloid precursor protein [20], a myriad of different mRNAs have been targeted with different ON analogues conjugated to CPPs [21]. Successful delivery of antisense ONs *in vivo* using CPPs was demonstrated for the first time with an antisense peptide nucleic acid (PNA) complementary to human galanin receptor 1 mRNA coupled to transportan or penetratin that specifically downregulated these receptors in rat [22]. Interestingly, although decoy ONs have been used extensively to modulate the activity of various transcription factors in different disease models, only three reports have emerged on successful vectorization of decoy ONs with CPPs [23-25].

As mentioned above, SCOs, negatively charged or uncharged ON analogues, have shown promising therapeutic potential in redirecting splicing in various diseases, and at least one clinical trial for treatment of Duchenne muscular dystrophy is continuing [26]. However, the needed doses of SCOs to obtain therapeutic effects are usually very high and the commonly used delivery methods for such ONs suffer, as already pointed out, from several drawbacks. Therefore, as CPPs are increasingly used for transport of a variety of cargos, they have also been applied for the delivery of different types of SCO, such as those based on PNAs, phosphorodiamidate

Table 1. Selection of CPPs applied in delivery of siRNA, decoy ON and SCOs.

CPP	ON type	Targeted mRNA	Cell line/animal	Ref.
siRNA as cargo				
Pen	-	SOD1, Caspase-3, -8, -9	Primary neurons	[40]
Tat	-	EGFP, CDK9	HeLa	[41]
Pen, TP	-	GFP, luciferase	C166-GFP, EOMA-GFP, CHO-AA8-Luc	[42]
Tat-U1A RBD	-	EGFR, dEGFP	A431, dEGFP-CHO	[46]
Tat-DRBD	-	dGFP, dRFP, luciferase	Primary cells, luciferase mouse model	[47]
Decoy ONs as cargo				
TP, TP10	-	NFκB	Rinm5F	[25]
TP10	-	Myc	N2a, MCF-7	[24]
SCOs as cargo				
Tat	PMO	Luciferase	HeLa L6, Hela pLuc 705	[32]
Tat, TP, Pen	PNA	Luciferase	HeLa pLuc 705	[29]
M918	PNA	Luciferase	HeLa pLuc 705	[33]
Tat	PNA	Luciferase	HeLa pLuc 705	[27,66]
MAP	PNA	Luciferase	HeLa pLuc 705	[67]
$(RXR)_4$	PMO	Dystrophin	DMD canine myoblasts	[27,34]
Pip2	PNA	Luciferase, dystrophin	HeLa pLuc 705, DMD mouse model	[68]
B peptide, P007	PMO	Dystrophin	DMD mouse model	[35-37]
R6-Pen	PNA	Luciferase	HeLa pLuc 705	[28]

CDK9: Cyclin-dependent kinase 9; CHO: Chinese hamster ovary; CPPs: Cell-penetrating peptides; dEGFP: Destabilized enhanced green fluorescent protein; dGFP: Destabilized green fluorescent protein; DMD: Duchenne muscular dystrophy; DRBD: Double-stranded RNA binding domain; dRFP: Destabilized red fluorescent protein; EGFP: Enhanced green fluorescent protein; EGFR: Epidermal growth factor receptor; MAP: Model amphipathic peptide; ON: Oligonucleotide; Pen: Penetratin; PMO: Phosphorodiamidate morpholino oligomers; PNA: Peptide nucleic acid; RBD: RNA binding domain; SCO: Splice correcting oligonucleotide; SOD: Superoxide dismutase; TP: Transportan.

morpholino oligomers (PMOs) and 2′-*O*-methyl-modified RNAs (2′-OMe ONs) [27-29].

In 1998, Kole and co-workers developed a functional splice correction assay for evaluating the relative delivery efficacy of different vectors for SCOs [30]. This assay is based on the HeLa pLuc 705 cell line that is stably transfected with a luciferase coding gene interrupted by a mutated β-globin intron. This mutation causes aberrant splicing of luciferase pre-mRNA, resulting in synthesis of non-functional luciferase. Masking the aberrant splicing site with an SCO redirects splicing towards the correct mRNA and consequently restores luciferase activity. This positive read-out assay has been used extensively for evaluating the delivery efficacy of various CPPs (**Table 1**). Early studies mainly used classical CPPs, such as Tat and penetratin [31,32], whereas later several other CPPs, such as M918 [33], $(RXR)_4$ [34] and R6-penetratin [28], were found to be more potent for this application. Moreover, several research groups have successfully applied CPP-conjugated SCOs to restore dystrophin expression *in vivo* (**Table 1**) [34-37]. They used murine models of muscular dystrophy and demonstrated, after intravenous injection of CPP–PMO conjugates, widespread correction of dystrophin expression in skeletal as well as in cardiac muscle. These studies clearly illustrate the clinical potential of CPPs for systemic delivery of SCOs to manipulate splicing patterns.

Despite great progress in the field of siRNA, only a handful of papers have reported on successful CPP-facilitated siRNA delivery (**Table 1**). The group of Gilles Divita was first to report on successful delivery of siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the CPP, MPG, non-covalently complexed with the siRNA [38]. Another excellent study published recently by Kumar and co-workers used a peptide derived from rabies virus glycoprotein (RVG) as a brain targeting molecule, shown to bind selectively nicotinic acetylcholine receptor, coupled to an all-D-Arg9 CPP through a glycine linker [39]. They applied this peptide for antiviral siRNA delivery into the central nervous system through a simple co-incubation approach and could prolong the survival of Japanese encephalitis virus-infected mice

Table 2. Covalent conjugation versus non-covalent complex formation in nucleic acid delivery.

Covalent strategy	Non-covalent strategy
Difficult to synthesize/conjugate	No extra synthesis needed
High cargo concentration needed	Low concentration of cargo needed
Suitable for neutral ONs such as PNA and PMOs	Applicable only for negatively charged ONs
Cargo-peptides have a defined stoichiometry	Difficult to generate and characterize homogenous particles
Not applicable for plasmid delivery	Applicable for plasmid delivery
Conjugated peptide can interfere with the activity of cargo	Free peptides might neutralize the ON

ON: Oligonucleotide; PMO: Phosphorodiamidate morpholino oligomers; PNA: Peptide nucleic acid.

without inducing any inflammatory cytokines or antipeptide antibodies. While the studies mentioned applied a simple co-incubation strategy, successful delivery of covalently conjugated CPPs to siRNAs by means of a disulfide bridge has also been reported [40-42]. Conversely, modest effects with CPP–siRNA conjugates have been reported by others [43,44]. It is apparent that the CPP–ON conjugation strategy plays a critical role in the outcome of the experiment and therefore this issue is discussed in more detail in the following section.

2.2 Vectorization strategies of oligonucleotides with cell-penetrating peptides

Principally, there are two possibilities to vectorize ONs with CPPs; either to use a covalent conjugation approach where the ON and CPP are connected by means of covalent bonds of different types or to use a simple co-incubation procedure where CPPs are allowed to form non-covalent complexes with ONs by means of electrostatic and hydrophobic interactions. The major difference between the successful [40-42] and unsuccessful studies [43,44], mentioned in the previous section, in delivery of siRNA covalently conjugated to CPPs was that constructs were not purified after conjugation in the three successful studies, whereas they were purified in the last two. Most probably there was free, unconjugated siRNA and peptide in the reaction mixture in the first studies, which could form non-covalent complexes and internalize to cells. Hence, siRNA covalently conjugated to CPPs were probably not able to mediate gene silencing. These results indicate that non-covalent co-incubation might be preferred over covalent conjugation, at least when using negatively charged double-stranded siRNAs. Furthermore, significantly higher concentrations of siRNA are usually needed when utilizing covalent conjugates in order to obtain a biological effect; also, the conjugation and subsequent purification steps make it considerably more laborious compared with a non-covalent approach. However, a major advantage with using covalent conjugates is that they have a defined molecular mass, which is preferable if they are to be used clinically. A list of pros and cons of the two different strategies is given in **Table 2**.

Although the co-incubation strategy might appear preferable over the use of covalently conjugated CPP–ON conjugates, aggregation between CPPs and ONs is still a major concern. In most cases it seems that the anionic ONs inactivate the cationic CPPs [45]. Indeed, CPP/ON complexes are effectively internalized when measuring quantitative uptake of fluorophore labeled ONs; however, in many cases they are unable to induce any biological activity.

To solve the issue of aggregation and CPP neutralization, two groups have recently presented a completely new CPP-based platform for the delivery of siRNAs [46,47]. In this system a Tat peptide fusion protein with an RNA binding domain (RBD) is utilized. In that way the RBD domain is used as a molecular glue to attach the Tat peptides to the siRNA. However, the two groups applied slightly different set ups. Ohtsuki and co-workers used a Tat-U1A RBD fusion protein where Tat-U1A RBD binds to a specific RNA sequence that has to be introduced to the siRNA of interest [46]. The platform reported by Eguchi and colleagues uses a double-stranded RBD carrying three Tat peptides (Tat-DRBD) that binds avidly to any double-stranded siRNA to confer shielding of the negative charges, thereby preventing peptide–RNA aggregation [47]. Moreover, Ohtsuki and co-workers had to photostimulate the internalized siRNA in order to release it from endosomes and induce gene silencing, whereas Eguchi and colleagues showed that the siRNA delivery was rapid, ubiquitous in the entire cell population and, most importantly, Tat-DRBD promoted significant siRNA-mediated gene silencing in different primary cell cultures without any photostimulation. However, in order to induce RNAi, rather high siRNA concentrations were used (100 – 400 nM), which is at least one log higher than with other commercial reagents. Also, the system is based on recombinant protein constructs that in many cases are very cumbersome to express and purify from bacteria. These results show that the CPP technology is feasible for siRNA delivery, but also that there is space for improvements.

3. Chemically modified cell-penetrating peptides for gene delivery

One main consideration when using CPPs for cargo delivery is their cellular fate and, hence, the effects on the bioactivity of the cargo. As mentioned above, CPPs are taken up primarily by endocytic pathways, and consequently the cargo is commonly retained in endosomes without reaching either the cytoplasm or any other cellular compartment, such as the nucleus. *In vitro* this obstacle can be overcome by using

Table 3. Chemical modifications of CPPs for non-covalent gene delivery.

CPP	Cargo	Biological effect	Cell line/animal	Ref.
N-terminal stearylation				
Arg8	Plasmid	Plasmid expression	Cos-7, NIH/3T3	[57,58]
Arg8	siRNA	Gene silencing	Primary rat neurons	[60]
TP10, Arg9, Pen	PS 2′-OMe ON	Splice correction	HeLa pLuc	[45]
C-terminal cysteamidation				
MPG, MPGΔ^{NLS}	Plasmid, siRNA	Plasmid expression, gene silencing	HS-68, Cos-7, HeLa	[38]
Pep-3	HypNA-PNA	Tumor growth inhibition	PC3 tumor-bearing mouse model	[69]
CADY	siRNA	Gene silencing	U_2OS, HUVEC, 3TC3, THP1	[51]
C-terminal cholesteryl modification				
All-D Arg9	siRNA	Tumor growth inhibition	CT-26 tumor-bearing mice	[55]
Histidine and/or cysteine modification				
EB1	siRNA	Gene silencing	HeLa, HepG2	[48]
Tat	Plasmid	Plasmid expression	U251, H4, T98G, C6, rat brain	[50]
Decanoic acid modification				
Tat	PNA	Splice correction	HeLa pLuc 705	[56]

CPPs: Cell-penetrating peptides; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Pen: Penetratin; PS 2′-OMe ON: Phosphorothioate 2′-*O*-methyl RNA; TP10: Transportan 10.

different lysosomotropic agents, for example chloroquine, but this approach is not suitable for *in vivo* applications.

Recently, several studies have emphasized the successful applicability of modified CPPs for delivery of nucleic acids by means of a non-covalent co-incubation strategy (**Table 3**). In order to promote endosomal escape and increase the transfection efficiency of CPPs, many different strategies have been used. One example is the development of a histidine-containing endosomolytic α-helical penetratin analogue, EB1, which was able to form complexes with siRNA and promote endosomal escape [48]. It was speculated that the successful breakout from the endosomes emanated from the formation of a helical structure on protonation in the acidic environment. Others have conjugated fusogenic peptides derived from viruses, for example HA2-peptide, to CPPs in order to facilitate release of oligonucleotides from endosomes [29,49]. In another study histidine and cysteine residues were incorporated into the Tat peptide sequence to facilitate endosomal release of plasmid DNA as well as to protect the cargo in the extracellular environment [50]. Also, the presence of a C-terminal cysteamide (**Figure 1D**) appears to be crucial for CPP-mediated siRNA delivery using the MPG [38] and CADY peptides [51]. This modification is believed to increase membrane association [52] and stabilize complex formation [53] by the formation of peptide dimers. Very recently, thiol-containing CPP conjugates have been shown to bind to cellular membranes and subsequently internalize more efficiently than peptides lacking thiols, which could explain the increased delivery using the cysteamide approach [54]. Also, cholesteryl (**Figure 1A**) modification of all-D-Arg9 has shown to improve siRNA delivery [55]. In a mouse model bearing a subcutaneous tumor, local administration of cholesteryl-modified peptide complexed with siRNA targeting VEGF led to tumor growth inhibition.

Another interesting strategy used to increase the bioactivity of CPP/ON complexes is to modify the particles with fatty acids. The group of Peter Nielsen recently reported that Tat-PNA-mediated splice correction increased by up to two orders of magnitude when conjugating decanoic acid (**Figure 1C**) to the conjugates [56]. Moreover, stearylation (**Figure 1B**) of CPPs has proved to be another powerful methodology to increase the transfection efficiency of plasmids [57,58], siRNA [59,60] and phosphorothioate 2′-OMe RNA [45] through a non-covalent co-incubation approach, resulting in the formation of nanoparticle complexes. Although the first three studies reported that stearylation of poly-arginine greatly increased CPP-mediated transfections, we were unable to observe any effect of the stearyl modification on the performance of either polyarginine or penetratin for the delivery of SCOs [45]. However, stearyl modification had a major impact on the amphipathic CPP, TP10, on the delivery of SCOs [45]. In previous studies, CPP/ON complex treatments have mostly been performed in serum-free media; however, stearylated TP10/2′OMe ON complexes have been shown also to preserve the transfection efficiency in serum-containing medium [45]. Collectively, these studies suggest that there are several ways to improve CPP-mediated delivery by introducing various chemical modifications that allow application of significantly lower ON concentrations.

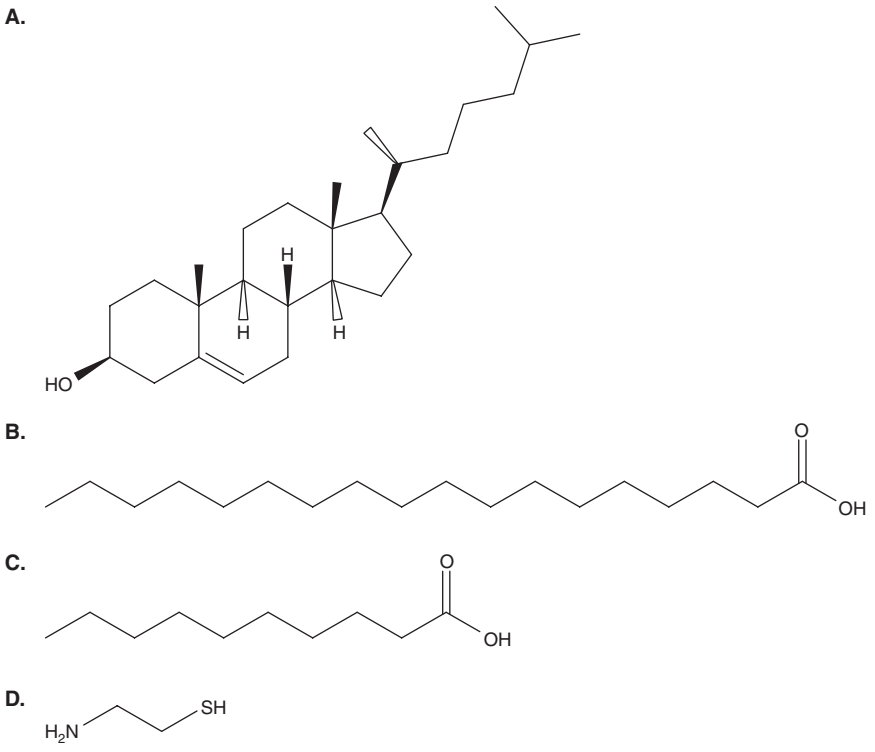

Figure 1. Structures of chemical entities used for cell-penetrating peptide modification in order to improve their delivery properties. A. Cholesterol. **B.** Stearic acid. **C.** Decanoic acid. **D.** Cyteamine.

4. Conclusion

Over the past 10 years, numerous studies have implicated the utility of CPPs for nucleic acid delivery both *in vivo* but mostly *in vitro*. These peptides are very versatile and have been exploited for the delivery of different classes of ONs in different preclinical settings, including models of cancer, stroke, heart disease and viral infections, with great success. Their non-toxic nature, especially compared with liposome-based delivery vectors, in combination with their relatively high delivery efficacy make them highly interesting for future therapeutic use. However, a concern with the use of unmodified CPPs for the delivery of ONs is, as mentioned, their frequent accumulation in inaccessible endosomal compartments. Hence, high concentrations of conjugates are generally needed to obtain significant biological responses. Although this is not necessarily a problem from a toxicity point of view, it requires up-scaling of synthesis if they are to be applied *in vivo* and cumbersome conjugation procedures, which makes it all very cost-inefficient.

By introducing chemical modifications such as fatty acids or conjugations to fusogenic virus-derived peptides, the bio-availability of CPP/ON complexes is increased dramatically as a result of improved endosomal escape, and significantly lower doses can therefore be applied (**Figure 2**). CPPs appear to be very promising for the delivery of various nucleic acids; however, the development of more efficient peptides is a prerequisite in order to progress into the clinics.

5. Expert opinion

Since the initial discovery of the first CPP, penetratin in 1994 [2], hundreds of new peptides have emerged with reported cell-penetrating properties. The underlying factors that have attracted attention to these peptides and their development are that many of them do not seem to have any size restraints in terms of cargo that can be transported into cells. Furthermore, they are believed to enter each and every cell in a population and there appear to be no cell type preferences for internalization. Thus, their versatility in combination with the ubiquitous delivery has laid the foundation for intense investigations aimed at resolving their uptake mechanism into cells. From the early view these peptides transduce cells in an energy-independent manner, most researchers in the field now agree that most CPPs enter cells by means of endocytosis, at least when conjugated to a cargo. An example is depicted in **Figure 3A**, where it is shown that PNA covalently conjugated to the CPPs TP10 and M918 promotes splice correction in the previously described HeLa pLuc 705 cell assay. When co-adding the lysosomotrophic agent chloroquine, splicing significantly increases, which confirms an endocytic uptake mechanism. However, a concentration of 5 μM conjugate is needed to

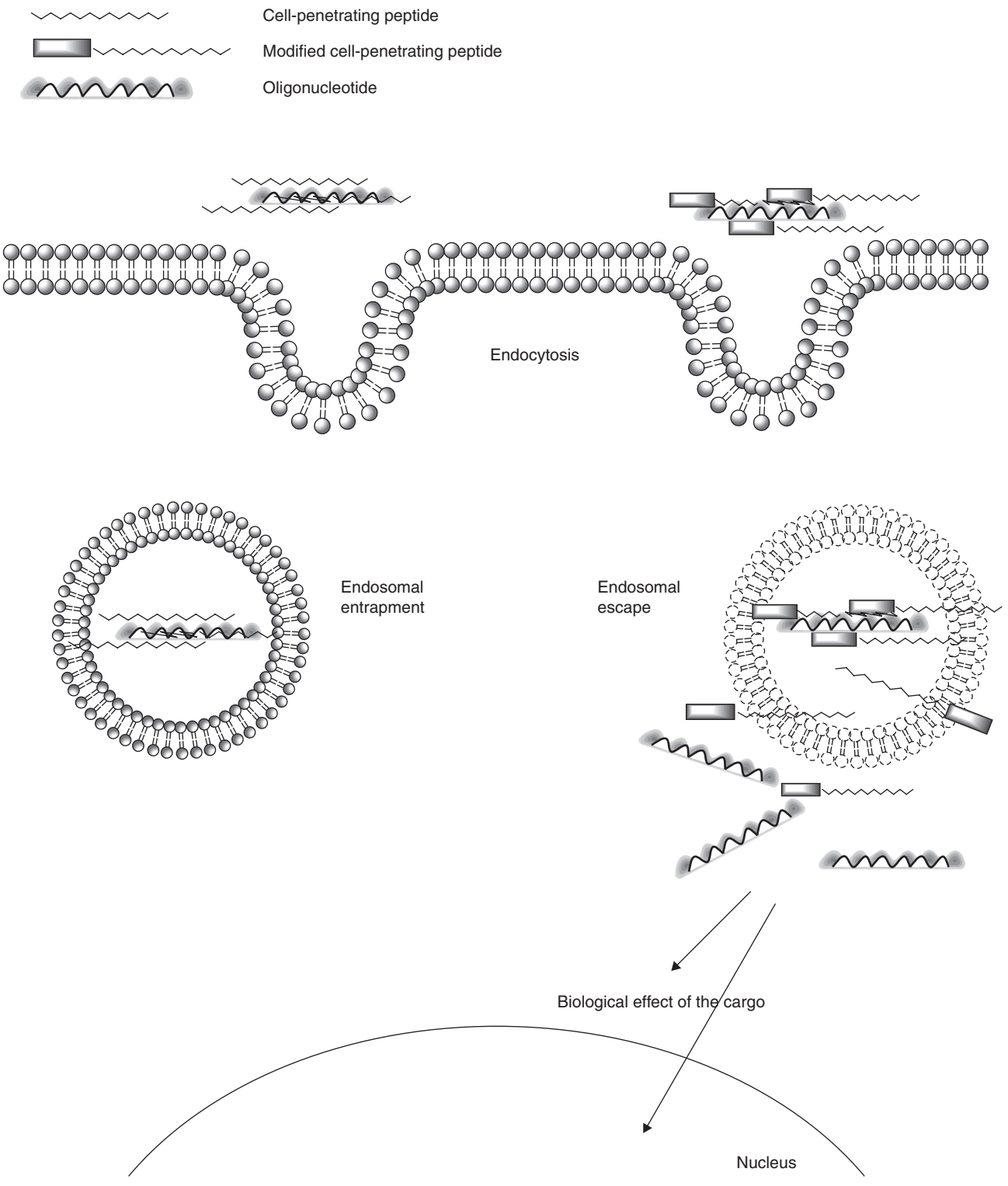

Figure 2. Schematic drawing illustrating the intracellular fate of ON/CPP complexes. In most cases, ON/CPP complexes bind to heparane sulfate proteoglycans on the surface of cells and subsequently induce endocytosis. Most internalized complexes remain entrapped in endosomal/lysosomal compartments, which greatly reduces the bioavailability of ONs. By introducing different chemical modifications, such as a stearic acid moiety to the CPP, the release of ONs from endosomes is significantly increased, thereby dramatically increasing the biological response.

CPP: Cell-penetrating peptide; ON: Oligonucleotide.

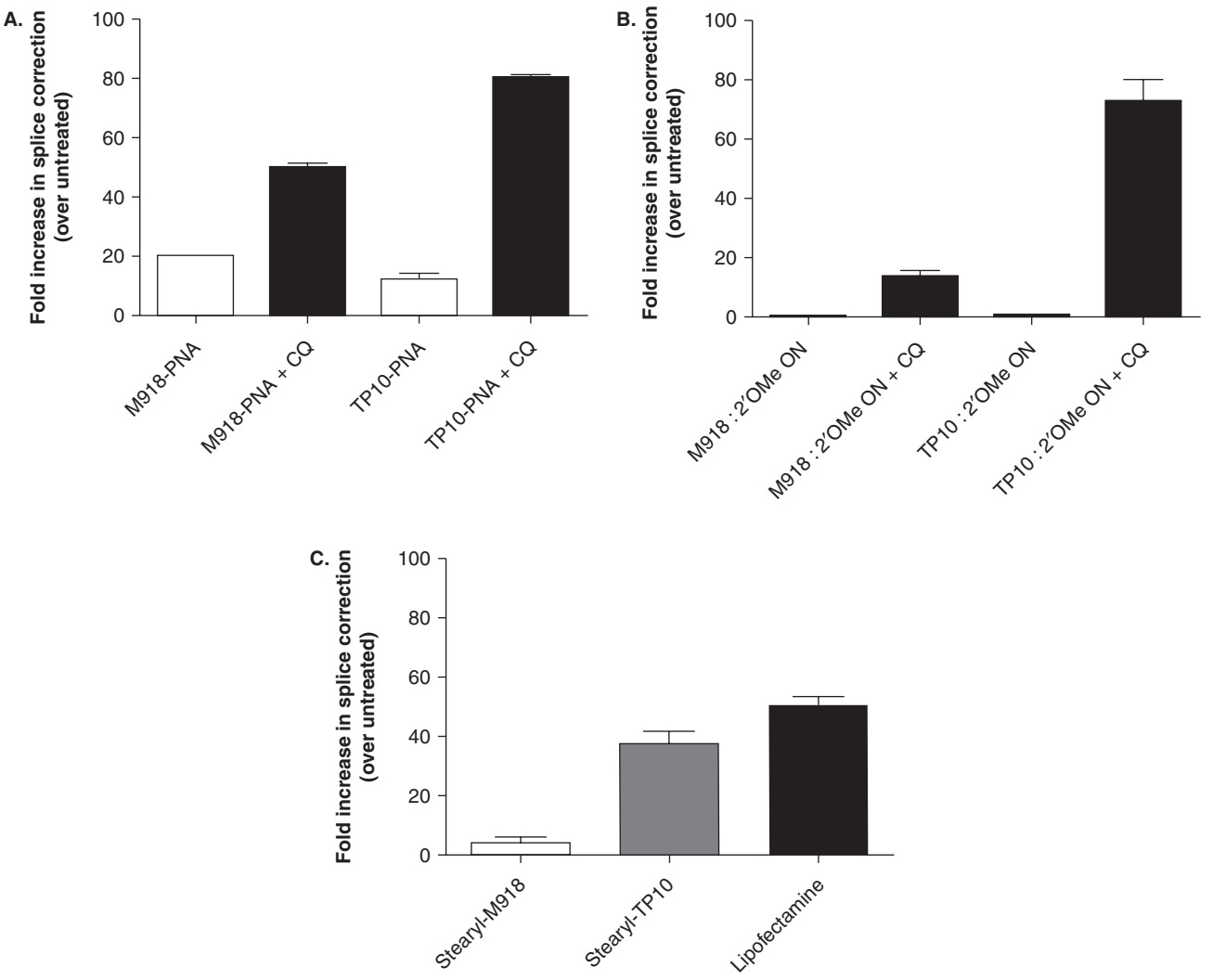

Figure 3. CPP-mediated splice correcting oligonucleotide delivery. A. Significant splice correction is observed 24 h after treatment with PNA covalently conjugated to two different CPPs at 5 μM concentration. Clearly, the conjugates are partially retained in endosomes because splice correction is increased significantly following treatment with the lysosomotrophic agent CQ. **B.** Unmodified CPP in complex with 200 nM PS 2′-OMe ON at a molar ratio of 10 is unable to promote splice correction unless co-incubated with CQ. This clearly illustrates that all non-covalent complexes when using unmodified CPPs reside exclusively in endosomes. **C.** When utilizing a stearylated TP10 in complex with PS 2′-OMe ON under the same conditions as in **B**, splice correction is drastically increased, reaching values on parity with the commercial agent Lipofectamine™ 2000. The stearylation approach is by no means universal because the same modification of M918 has no effect even though M918 is more active when using covalent conjugates, as shown in **A** (EL Andaloussi, M Mäe and Ü Langel, unpublished data).

CPP: Cell-penetrating peptide; CQ: Chloroquine; ON: Oligonucleotide; PNA: Peptide nucleic acid.

obtain such effects, and if the aim is to use these compounds systemically *in vivo*, very large amounts of both CPP and PNA are needed, thus making it very cost-inefficient.

Various means of improving ON delivery with CPPs have been tested. By using CPPs in combination with existing transfection reagents such as cationic liposomes and polycations, transfections have increased dramatically for plasmids and ONs [61-63]. Others have used more sophisticated delivery systems consisting of multifunctional liposomes carrying unmodified or modified CPPs both *in vitro* and *in vivo*, with great success [64,65]. Although these systems are highly efficient, it is laborious to construct such complex vectors. Also, there are clearly issues related to potential toxicity of liposome-based vehicles.

One very potent vector for the delivery of SCOs, which should also be applicable for other types of ON as well, is the recently described stearylated-TP10. The authors have previously assessed the potential of 30 different CPPs to convey an SCO based on 2′-OMe ON chemistry as non-covalent complexes in the above-mentioned splice correction assay. None of them was able to promote any significant biological response. However, as seen in **Figure 3B**, which is in line

with most other tested CPPs, CPP/SCO complexes promote significant pre-mRNA correction after co-treatment with chloroquine. This suggests that the complexes are internalized but remain trapped in endosomal compartments. By introducing a stearic acid moiety, the effect of TP10 was increased dramatically (**Figure 3C**). In fact, this peptide is almost as efficient as Lipofectamine™ 2000 (Invitrogen, Sweden) *in vitro* and substantially less toxic. Interestingly, the effect of stearylation is by no means universal as it has very little impact on the activity on M918 or any other tested peptide, including penetratin and oligoarginine [45]. These results clearly demonstrate the complexity of CPPs, but they also show that by chemically modifying certain CPPs, their delivery properties can be drastically improved. By using stearyl-TP10 in a simple co-incubation setting, devoid of cumbersome chemical conjugations, it is possible to use SCO concentrations that are 25 times lower compared with using covalent conjugates, and still obtain stronger biological effects.

It is clear that CPP technology holds great potential for ON delivery in various settings. Although most studies so far have been conducted *in vitro* and more extensive *in vivo* testing is required that addresses the pharmacokinetic and pharmacodynamic properties, it certainly looks promising.

Declaration of interest

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