Advanced Drug Delivery Reviews 106 (2016) 172–182

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Peptides for nucleic acid delivery☆

Taavi Lehto [a,b,*], Kariem Ezzat [a], Matthew J.A. Wood [c], Samir EL Andaloussi [a,c]

[a] Department of Laboratory Medicine, Karolinska Institute, Stockholm SE-171 77, Sweden
[b] Institute of Technology, University of Tartu, Nooruse 1, 50411 Tartu, Estonia
[c] Department of Physiology, Anatomy, and Genetics, University of Oxford, OX13QX Oxford, United Kingdom

ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form 8 June 2016
Accepted 15 June 2016
Available online 25 June 2016

Keywords:
Cell-penetrating peptides
Nucleic acid delivery
Oligonucleotides
Antisense oligonucleotides
siRNA
Nanoparticles
Splice-switching oligonucleotides
Delivery peptides

ABSTRACT

Nucleic acids and their synthetic oligonucleotide (ON) analogs are a group of gene therapeutic compounds which hold enormous clinical potential. Despite their undoubted potential, clinical translation of these molecules, however, has been largely held back by their limited bioavailability in the target tissues/cells. To overcome this, many different drug delivery systems have been devised. Among others, short delivery peptides, called cell-penetrating peptides (CPPs), have been demonstrated to allow for efficient delivery of nucleic acids and their ON analogs, in both cell culture and animal models. In this review, we provide brief overview of the latest advances in nucleic acid delivery with CPPs, covering the two main vectorization strategies, covalent conjugation and nanoparticle formation-based approach. In conclusion, CPP-based drug delivery systems have the capacity to overcome the hurdle of delivery and thus have the potential to facilitate the clinical translation of nucleic acid-based therapeutics.

Contents

1. Introduction

Our expanding knowledge in genetics, gene expression regulation, and availability of many excellent genetic tools has rendered principally the whole genome and its products amenable for therapeutic intervention with nucleic-acid-based molecules. In the light of this, many gene therapeutic approaches have arisen, from classical gene replacement therapy to more novel antisense-based methods, aiming at altering gene expression by various means, including by expressing/correcting deficient genes, interfering with transcription, pre-mRNA splicing, mRNA catabolism, or translation. Collectively, different classes of nucleic acids and their synthetic ON analogs hold enormous clinical potential for the treatment of disease.

The most basic approach is to increase the expression of a gene of interest by incorporating it into viral vectors or bacterial plasmids and expressing it in tissues/cells to restore normal or correct for pathologic

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Biologically-inspired drug delivery systems".

* Corresponding author at: Department of Laboratory Medicine, Karolinska Institute, 141 57 Huddinge, Sweden.
E-mail address: taavi.lehto@ki.se (T. Lehto).

http://dx.doi.org/10.1016/j.addr.2016.06.008

gene expression, i.e. so-called classical gene therapy approach. Lately, the gene therapy arsenal has been complemented with a wide variety of strategies exploiting short oligonucleotides (ONs). These mostly antisense-based methods are unique, as they are highly specific and can be used to target virtually any single or group of genes. Antisense methodologies include classical antisense ONs (ASOs), splice-switching ONs (SSOs), antigene ONs, small interfering RNAs (siRNAs), microRNAs (miRNAs), anti-miRNAs (antimiRs) (as further described in many excellent reviews [1–6]). Typically, effects of such compounds lead to enhanced target gene expression, modulation/conversion between the different gene products, or silencing of specific genes. Recently, also several additional gene therapeutic platforms have emerged, such as gene-editing platforms (ZFNs, TALENs, CRISPER/CAS9) for accessing and altering the genomic DNA, but also chemically stabilized mRNAs that could be used as templates for protein synthesis (see reviews on gene editing techniques in Refs. [7,8] and on modified mRNA in Ref. [9] for further information). Collectively, these molecules have allowed to significantly expand the space of pharmacologic targets otherwise undruggable with conventional drugs.

The common denominator between all nucleic acid-based molecules is that they are based on DNA or RNA and/or their synthetic analogs. These biopolymers have very high molecular weight and, with few exceptions, are highly charged, which severely restricts their ability to freely cross cellular membranes and reach their active sites within the cells. Moreover, the intracellular site-of-action for different nucleic acid molecules resides in various intracellular compartments. Hence, molecules which have their active site in the nucleus, e.g. SSOs/plasmids, have to additionally cross the nuclear membrane. Consequently, nucleic-acid-based molecules in their naked form have very limited bioavailability [10,11]. It is widely recognized that for clinical translation, most of the nucleic acid-based molecules would require assistance in the delivery. To address this "delivery issue," a variety of different drug delivery systems have been developed.

Delivery of nucleic acids can be divided into two main strategies—viral and non-viral delivery. Viral vectors are exceptionally efficacious in delivering genetic material to cells, as millions of years of evolution have shaped and optimized them for this purpose. Recently, owing to the developments in vector design and safety, viral gene therapy approaches have made huge leaps toward the clinics for many genetic disorders (as recently reviewed in Ref. [12]). Depending on the type of vector, viruses will, however, always retain some of their inherent weaknesses, including potential immunogenicity, tumorigenicity, limited cargo-carrying capacity, complex production, etc. Importantly, viral vectors are not universally applicable for all nucleic acid-based molecules, since they are not compatible for example with the delivery of short synthetic ONs. Therefore, in the context of ON delivery, focus has always been on the development of non-viral delivery alternatives for viruses. Non-viral delivery vectors can be based on many different classes of compounds, including various cationic lipids, polymers, peptides, or carbohydrate analogs [13–15]. Among these, small delivery peptides, termed cell-penetrating peptides (CPPs), have gained considerable interest.

CPPs are cationic and/or amphipathic peptides, usually up to 30 amino acids in length, that are characterized by their remarkable ability to convey different cargo molecules into cells and tissues [16–18]. Importantly, they demonstrate excellent potential for the delivery of nucleic acids, ranging from natural or synthetic short RNA or DNA ONs to much larger plasmid molecules, both *in vitro* and *in vivo* [19–22]. CPPs can have very different origin and are in many ways ambiguous to define. Many early CPPs were derived from protein sequences that were found to have translocating properties. Subsequently, also peptide/protein engineering has been deployed, by combining domains with different properties (e.g. for adopting secondary structures, for avid nucleic acid binding, for enhanced membrane activity, and more) from various naturally occurring proteins (so-called chimeric CPPs). Furthermore, a growing body of information on the properties of CPPs has also led to the development of peptides with completely designed sequences. Recently, several algorithms have been built that allow for prediction of amino acid sequences that can potentially have translocating properties [23]. Taken together, independent of their origin or classification, the common denominator between all CPPs is their ability to translocate different cargo molecules across biological membranes to the interior of cells.

It is generally accepted now that CPPs, especially when associated with cargo molecules, are predominantly internalized to cells via energy-dependent mechanisms involving different sorts of endocytosis (see Fig. 1 for illustration on uptake pathways and trafficking events generally associated with CPPs) [24–27]. In this context, all major endocytic pathways, including macropinocytosis, clathrin- and caveolae-mediated endocytosis, have been described to be involved in the uptake of CPPs. A body of information also indicates that the specific endocytic pathways utilized by the CPPs are strongly dependent on various factors. For example, the route of uptake can be highly dependent on the properties of the cargo molecules, their relative concentrations, specific cell lines and/or tissues studied, etc. Moreover, at high concentrations and/or when blocking specific pathways, several endocytic routes can be used in parallel, or alternative pathways can compensate for the direct inhibition of another. As CPPs are internalized by endocytosis they are trafficking and, to a great extent, get sequestered in endo-lysosomal compartments. This so-called endosomal entrapment also serves as one of the key limiting factors in the bioavailability of CPPs [26,29]. Consequently, the activity of CPPs is to a high extent dependent on their ability to escape from the endo-/lysosomal compartments. As with other non-viral approaches, a significant part of the work with CPPs has concentrated on the development of strategies aiming at increasing escape of the compounds from endosomes. Successful approaches have included increasing the interaction capacity with membranes, including modification with hydrophobic compounds (e.g. fatty acids or cholesterol), fusogenic peptide sequences derived from endosomolytic domains of viruses (e.g. HA2 domain of the influenza virus A), incorporation of protonatable domains for buffering the endo-/lysosomes (e.g. chloroquine analogs or histidines), photoinducible entities, and more. While different approaches have proved to be effective, finding the right balance between the endosomolytic activity and the potential toxicity is crucial from a safety point of view, since these modifications generally increase the cellular toxicity. In addition to endocytosis, some membrane active peptides, such as MPG or CADY peptides, have also been reported to directly translocate cells and deliver their cargo, potentially avoiding endocytic pathway altogether [28].

Similarly to the uptake debate, divergent results have been presented as to which components the CPPs initially interact with at the plasma membrane. Generally, it is accepted that different cell surface proteoglycans, especially glycosaminoglycans (GAGs), such as heparan sulfates (HS), are responsible for the initial cellular association of CPPs. Recently, additional co-factors, such as scavenger class A (SCARA) receptors, have also been shown to be involved in the association and/or uptake of CPPs. Moreover, if CPPs are functionalized with targeting ligands, association and uptake could be dependent on the mode-of-internalization of the particular receptor [11]. Recently, in the context of lipid nanoparticles, it has been shown that exocytosis and re-uptake can play an important role in uptake and trafficking of non-viral vectors [30], and this is an important aspect in potential (re-)uptake and (re-)distribution of CPPs that needs to be assessed in the future (see also Fig. 1).

Generally, there are two main strategies how CPPs can be used to vectorize ONs: by directly conjugating ONs to the CPPs (covalent conjugation approach) or by forming non-covalent nanoparticles between the CPPs and ONs (nanoparticle-based approach). An important advantage of the chemical conjugation approach is that it yields a CPP-ON molecule with defined structure and stoichiometry [31,32]. However, this approach is in principle restricted to the vectorization of charge-neutral ONs, including peptide nucleic acid (PNA) and phosporodiamidate

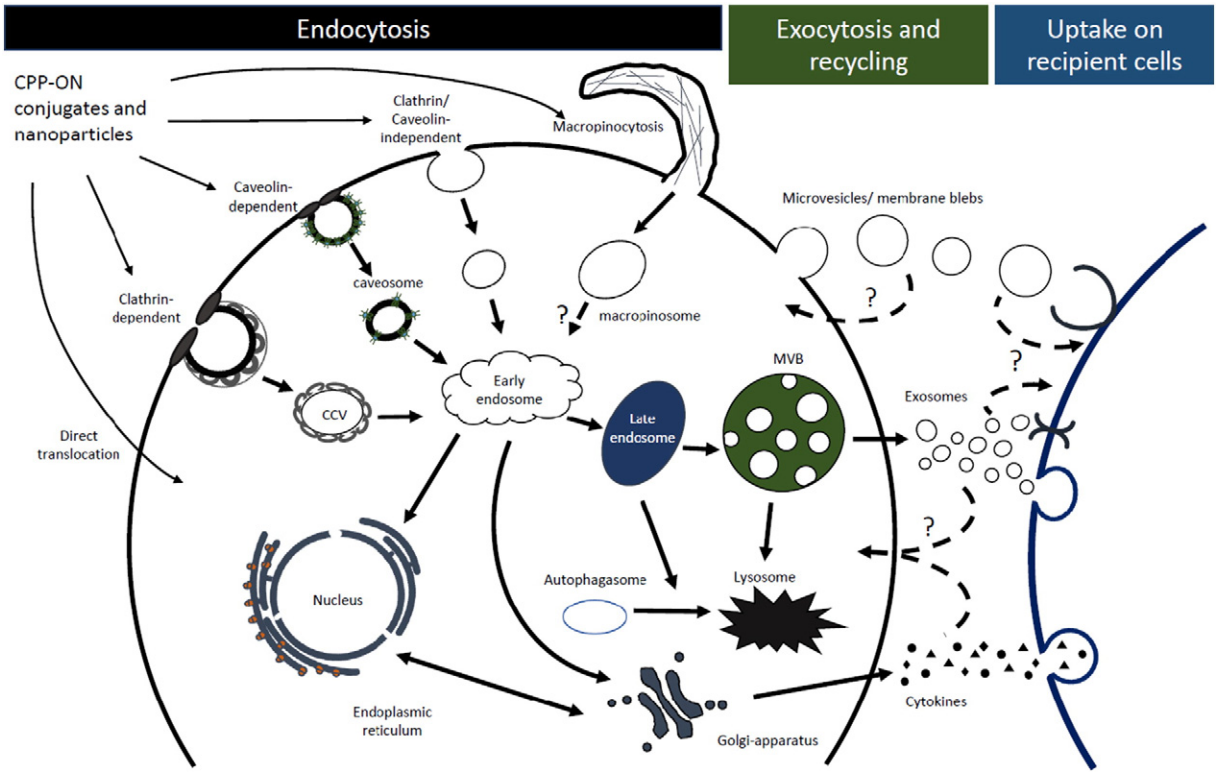

Fig. 1. *Uptake and trafficking pathways of CPPs.* CPPs use predominantly different sorts of endocytosis to gain access to the interior of cells. In the context of this, all major pathways for CPP uptake have been described, including clathrin- and caveolae-mediated endocytosis, as well as micropinocytosis. Less information is available for other less-defined pathways, such several clathrin- and caveolae-independent endocytosis mechanisms. Uptake of the cargo molecule is followed by complex intracellular trafficking events toward early/sorting endosomes, late endosomes/multivesicular bodies (MVBs), lysosomes or Golgi network. Typically, endo-/lysososmal maturation is characterized by gradual drop in the pH. Of note, recycling pathways can direct cargo also through late endosomes/MVBs for being released to extracellular milieu via extracellular vesicle (EV) release. In this case, the cargo could become incorporated into exosomes and subsequently be taken up by other cells (re-distribute) or by the same cell (re-uptake). In addition to the more dominant endocytic pathways, some membrane active CPPs have also been reported to be taken up by direct physical translocation over the cellular membrane, which potentially allows them to avoid endocytic pathways altogether.

morpholino (PMO) chemistries. Although, conjugates with charged ONs have been, and are still, reported in the literature, it is very complicated to purify such conjugates and, even when purified, they are prone to form complexes due to the charge interactions of the anionic ONs and cationic CPPs.

The nanoparticle-based approach on the other hand is applicable to a much wider repertoire of ON chemistries and can be applied also for the vectorization of larger nucleic acids molecules, such as plasmids [33,34]. Nanoparticle formation between cationic CPPs and anionic nucleic acid cargoes is mainly driven by electrostatic and hydrophobic interactions (and to a certain extent by hydrogen bonding). Surprisingly, until recently, only a limited set of CPPs has been reported to allow efficient vectorization of nucleic acids using a nanoparticle-based approach. The main reason for this is that while most of the CPPs would form nanocomplexes/nanoparticles with nucleic acids, the particles formed are too unstable and very easily dissociate in the biological fluids and have limited capacity for endosomal escape. Consequently, the CPP vectors that have been shown to display delivery potential in the form of nanoparticles usually comprise some additional chemical modifications in their structure. Compared to their CPP/ON conjugate counterparts, nanoparticle-based formulations, however, are more heterogeneous and less-defined in their nature.

CPPs are also an excellent platform for further modifications with different functional domains. These could include units for providing stealth-properties (e.g. PEGylation), targeting ligands for providing enhanced tissue-specific accumulation, modifications that allow for endosomal release, etc. As CPPs are assembled on a solid-support, different orthogonal protection strategies permit the introduction of functional groups in exactly defined locations in the primary CPP sequence. Additionally, it is possible to introduce functionalization domains on the already formed nanoparticles by using so-called post-PEGylation or post-targeting strategies. Importantly, while functionalization approaches have been successfully used in many applications in the context of CPPs, including PEGylation and targeting strategies, they have until now received only limited attention in general.

In this review, we do not aim to exhaustively cover all the literature on CPP-based nucleic acid delivery but provide a brief overview of the latest advances in using CPPs for nucleic acid delivery. Importantly, we will concentrate on the approaches, both in the form of direct conjugates and nanoparticles, where CPPs act as a main drug delivery component. Hence, many strategies where CPPs are used rather as adjuvants, or components of more complex drug delivery systems, will not be covered.

2. Covalent conjugation approach for nucleic acid delivery with CPPs

The first successful CPP-ON conjugates were based on the PNA chemistry. In PNAs, the deoxyribose phosphate backbone is replaced by N-(2-aminoethyl) glycine linkage and the nucleobases are attached via a methylene carbonyl linkage to the glycine amino group [35]. Since PNAs have a neutral peptide-like backbone, it was easy to create CPP-PNA conjugates via continuous synthesis with the peptide. Several lysine-rich and arginine-rich CPPs have shown to enhance PNA uptake and activity as classical antisense ONs to reduce gene expression or as SSOs to manipulate splicing patterns. Sazani P. et al. have shown that adding 4 lysines to PNA significantly enhanced the activity for splice switching using a GFP based splice switching assay [36]. Bendifallah N. et al. have screened several well-known CPPs (Transportan, oligoarginine (R7–9), pTat, Penetratin, KFF, SynB3, and NLS) for their efficiency in mediating PNA uptake upon covalent conjugation [37]. In a

luciferase-based splice switching assay model, they found that the transportan 10 (TP10) conjugates had the highest activity. Similarly, we have shown previously that TP10 along with a few other CPPs are potent vectors for delivery of PNA-based SSOs in vitro; however, all conjugates were relatively sensitive to the presence of serum [38,39]. This sensitivity to serum might explain the mixed results of PPNAs in *in vivo* settings. Only a handful of reports have demonstrated PPNA activity *in vivo*. This was for example shown in mouse models of severe combined immunodeficiency (SCID) and for genetic targeting in hematopoietic progenitor cells in mice [40,41]. More recently, PPNAs demonstrated promising results as antimiRs, *in vitro* targeting mir-221 in breast cancer MDA cell lines, and targeting mir-155 in lymphoma mouse models [42,43]. This has renewed the interest in this chemistry as a promising therapeutic modality.

However, another class of CPP-ON conjugates is more widely used for *in vivo* applications, especially in the context of treating neuromuscular disease and bacterial infection, the so-called peptide-PMOs (PPMO). PMOs are chemically modified ONs that harbor a morpholino moiety instead of the ribose moiety and phosphorodiamidate linkages instead of the phosphodiester linkages. CPP conjugation to PMO has been performed via a variety of methods including maleimide linkage, disulfide linkage, click chemistry, or amide linkage between the free PMO secondary amine and an activated carboxylic group at the c-terminus of the peptide, which is the most popular [21]. Initial reports showing promising results using this chemistry were based on using arginine-rich peptides together with other chemical modifications such as aminohexanoic acid and beta-alanine [44,45]. The activity and stability of these conjugates in serum conditions and promising results *in vivo* led to development of several generations of PPMOs based on the same design concept and to their extensive screening in preclinical studies, especially in the context of Duchenne muscular dystrophy (DMD).

DMD is among the most common muscular dystrophy disorders affecting approximately 1 in every 3500 newborn boys [46]. It is caused by mutations in the *DMD* gene causing the generation of premature termination codons and/or out-of-frame transcripts [47]. It is characterized by progressive muscle degeneration normally leading to wheelchair dependence by the age of 12 and to premature death due to cardiac and respiratory failure before the age of 30. With no available therapy, exon skipping was considered one of the most promising approaches. SSOs can induce exon removal from the mature DMD transcript leading to the restoration of the mRNA reading frame and the production of a shorter version of the protein that is still functional [48]. Several clinical trials have tested this approach in patients using naked 2′-*O*-Methyl phosphorothioate (2′OMe) or PMO chemistries [49]. However, due to the poor uptake and biodistribution of these chemistries, the clinical trials have shown mixed results highlighting the need for better vectors for enhanced ON uptake.

PPMOs have been extensively tested in the *mdx* DMD mouse model which harbors a mutation in exon 23 encoding for a premature termination codon within the *DMD* gene. The $(RXR)_4$ peptide was the first CPP conjugate to be tested in the *mdx* DMD model and demonstrated high levels of exon skipping in different muscles [50]. Subsequently, another arginine-rich peptide, B-peptide, was shown to demonstrate high exon skipping in cardiac and skeletal muscles leading to an increase in muscle strength and prevention of cardiac failure induced by dobutamine stress [51]. In parallel, work from our group has shown that it is possible to improve efficacy of B-peptide by further fusing it with a muscle targeting peptide identified by phage display libraries (MSP peptide), resulting in up to 2–5-fold improvement over the activity of parent B-PMO [52].

More recently, a new series of CPPs for conjugated delivery of PMO termed the 'Pip' series have been developed. The CPPs of this series are characterized by a central hydrophobic core flanked on either side by arginine-rich sequences. Among the highly efficient members of this series are Pip5e–PMO which induced high levels of dystrophin restoration in various muscles including the heart following a single 25 mg/kg intravenous (IV) administration [53]. The more efficient Pip6a exhibited higher dystrophin splicing activity in heart over the Pip5e conjugate [54–56]. In order to understand the magnitude of efficiency of these constructs, it has to be emphasized that the dystrophin restoration levels achieved by these conjugates at doses less than 30 mg/kg after single injection can only be achieved by doses in the range of hundreds of mg/kg even for a very advanced chemistry such as tricyclo DNA and after repeated injection [57]. This shows that this class of molecules is very promising as a therapeutic; however, it requires further optimization and development regarding the toxicity and biodistribution profiles.

The success of the PPMO chemistry led to the expansion of their application to other genetic disease models and as antiviral and antibacterial agents. Recently, a CPP conjugated antisense ON strategy has been tested in a mouse model of the triplet repeat myotonic dystrophy leading to the neutralization of mutant RNA toxicity and elimination of myotonia [58]. This paves the way for the application of this technology in other genetic diseases such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS). B-PMO was also successfully used to restore the expression of Bruton's tyrosine kinase (BTK) by splice switching in a mouse model of X-linked agammaglobulinemia [59]. As antivirals, RXR-based CPP-PMO, targeting the start of the coding region of human respiratory syncytial virus (RSV), was shown to block viral replication *in vivo* both in mouse and porcine models of the virus [60,61]. They also displayed activity in reducing murine hepatitis virus (MHV) titers leading to protection from viral associated tissue damage in the liver [62]. Similar promising results using the same class of peptides were shown for dengue 2 virus (DENV-2) and West Nile Virus (WNV) [63,64]. Additionally, the potential of using CPP-PMO conjugates as antibacterial agents has also been explored. Arginine-rich RXR-based PMO conjugate targeting the *acpP* gene reduced bacterial blood titers and enhanced survival of mice infected with *E. coli* when administered 12 h after infection [65]. Furthermore, Wesolowski D. et al. have demonstrated that an arginine-rich peptide derived from human T-cells when coupled to PMO targeting gyrase enzyme, displayed potent antibacterial activity against a variety of pathogenic strains including *E. coli*, *K. pneumoniae*, and *S. aureus* [66]. Recently, several PPMO conjugates were shown to be highly effective against other pathogenic strains such as *Bacillus anthracis* and Acinetobacter strains [67,68]. With a growing problem of antibiotic resistance worldwide, the PPMO platform seems to be a promising alternative to conventional antibiotics.

Examples of CPP-ON conjugates and their application in different disease models are also described in Table 1.

3. Non-covalent nanoparticle-based approach for the delivery nucleic acids with CPPs

The nanoparticle-based approach for formulating nucleic acid, originating from the works with cationic lipids and polymers, was

Table 1
Examples of CPPs directly conjugated to oligonucleotides and their applications.

Peptide	Sequence	Application	Reference
Transportan-PNA	GWTLNSAGYLLGKINLKALAALAKKIL	Galanin receptor antagonist	[123]
B-PMO	RXRRBRRXRRBRXB	DMD	[51]
B-MSP-PMO	RXRRBRRXRRBRXB ASSLNIAX	DMD	[52]
Pip5e-PMO	RXRRBRRXR ILFQY RXRBRXRB	DMD	[53]
Pip6a-PMO	RXRRBRRXR YQFLI RXRBRXRB	DMD	[54]
T-cell-derived CPP-PMO	YARVRRRGPRGYARVRRRGPRR	Antibacterial	[66]
$(RXR)_4$-PMO	RXRRXRRXRRXR	Antibacterial	[68]
$(RXR)_4$XB-PMO	RXRRXRRXRRXRXB	Antiviral	[60,61]
K-PNA-K3	K-PNA-KKK	Antimir 155	[43]

B—beta-alanine, X—amino hexanoic acid.

introduced in the context of CPPs by several groups in the late 1990s [69,70]. The group of G. Divita pioneered many of the early reports on the utilization of CPPs using this approach. In several studies, chimeric 27-mer MPG peptide was used to complex short DNA, pDNA, and antisense ONs into nanoparticles and efficient delivery of these particles into mammalian cells was demonstrated [69,71]. Divita and colleagues also reported on an MPG-8 peptide and demonstrated its excellent delivery ability for siRNAs, both *in vitro* and *in vivo* [72]. In this study, MPG-8/siRNA nanoparticles, targeting cell cycle regulator cyclin B1, were shown to efficiently reduce tumor growth when injected intratumorally (IT) in tumor-xenograft mice. Interestingly, when studying the same formulation via systemic route, it remained completely inactive. To enhance *in vivo* stability, MPG-8 was further modified with cholesterol. The resulting Chol-MPG-8, used in combination with unmodified MPG-8 for formulation, was shown to provide systemic activity in two different tumor-xenograft models in mice. The effect of cholesterol was attributed to increase the serum half-life of the nanoparticles and enhanced penetration/distribution into the tumor tissue [72]. In parallel, this group has also reported on another interesting vector for siRNA delivery called CADY peptide [73]. Although no *in vivo* information is yet available with this peptide, several reports have indicated its considerable potential for the delivery of siRNAs in cell culture models.

From the CPP design point-of-view, these "MPG" peptides bear interesting features. First of all, they are amphipathic in character; however, later-generation peptides, such as MPG-8 and CADY, are able to also adopt/retain their helical structure in the presence of biological membranes (so-called secondary amphipathicity) [74]. Moreover, "MPG" peptides also carry additional modifications in their structure that are crucial for their activity. All "MPG" peptides are N-terminally acylated and harbor a cysteamide modification in the C-terminus. Introduction of such a thiol group stabilizes the nanoparticle by allowing crosslinking between the thiol groups on different CPP molecules within the nanoparticle. Similar stabilizing effects provided by thiols can be introduced by the addition of cysteines. Furthermore, adopting helical conformation seems to be a very beneficial feature for many effective CPPs that work in nanoparticle-based settings. Interestingly, helically locked conformation can be introduced by so-called stapling chemistry [75]. While, it has received very limited focus for CPPs, it is a very intriguing approach for future vector development (Gissberg et al., manuscript in preparation).

Another line of hydrophobic modifications are based on the addition of different fatty acids to the CPPs. In this context, especially addition of stearic acid or stearylation has been used with great success. Futaki et al. was to our knowledge the first to introduce this modification in CPPs [6]. In their early report, they showed the benefit of the modification on Arg8 peptide and demonstrated its applicability for plasmid transfections in cell culture models. Similarly, we have shown that stearic acid modification on the $(RxR)_4$ peptide transformed this CPP to be applicable for nanoparticle-based approach and displayed considerable delivery potential for plasmids and SSOs in cell culture models [76]. Interestingly, stearylated polyarginines *per se* are not usually very effective vectors, due to the limited stability of their particles, when used as a single component vector. However stearyl-Arg8 has been deployed as an adjuvant in liposomal formulation, such as so-called multifunctional envelope-type nanodevice (MEND), where it is utilized for core-particle formation with nucleic acid and also used on the surface for enhanced cellular uptake [77].

PepFect peptides are another intriguing group of amphipathic CPPs that have successfully deployed stearic acid modification and shown delivery potential for a wide variety of nucleic acid-based cargoes, including SSOs, siRNAs, and plasmids [78–83]. In the first generation, TP10 peptide was modified with stearic acid in its N-terminus, generating the PepFect3 (stearyl-TP10) peptide [78]. Several studies demonstrated the ability of PepFect3 to efficiently transport SSOs or pDNA. In the first report, Mäe et al. used PepFect3 for formulating 2′OMe SSOs and by using HeLa pluc705 splice correction model, they showed significant splice switching activity [78]. In the subsequent study, we extended the delivery properties of PepFect3 for plasmid DNA (pDNA) delivery and demonstrated effective gene delivery both *in vitro* and *in vivo* in mice [79]. Local administration of PepFect3/pDNA nanoparticles to the skin or muscle in mice induced around 10-fold increase in gene expression as compared to naked pDNA.

Subsequently, in search for delivery vectors for siRNAs, a small library of stearic acid analogs with different CPPs were generated and screened in different reporter cell lines, but unfortunately, all of the screened CPPs remained inactive for RNAi-mediated gene silencing. It was rationalized that these CPPs would require further endosomolytic properties for enhanced endosomal release to effectively reach to the cytoplasm. For this, we designed a novel endosomotropic modification, trifluoromethylquinoline (TFMQ) moieties (a chemical analog of the endosomotropic agent chloroquine), and modified the previously reported PepFect3 peptide with several TFMQ molecules, producing the PepFect6 peptide [80]. PepFect6 proved to be a very potent vector for siRNA delivery. The potency of PepFect6 was assessed in many different cell culture models, including T-cells, HUVECs, and mouse ES cells, and it was more effective than commercially available transfection agents. Importantly, the activity of PepFect6-mediated delivery of siRNA was further corroborated in several *in vivo* models for gene silencing.

Another interesting vector arising from the PepFect family is the PepFect14 peptide. In PepFect14, changes in the amino acid sequence of TP10 were carried out, most prominently introducing ornithines instead of lysines as a source of positive charge. Interestingly, the PepFect14 peptide proved to be a very effective vector for many nucleic acid- and ON-based molecules, including SSOs, siRNAs and pDNA [81–83]. An important finding using PF14 was that it could be formulated with SSO in the presence of different pharmaceutical excipients and dried as solid formulations and be re-used several months later with maintained activity [81]. In follow-up work, it was demonstrated that also siRNA could be formulated in the same manner and proved to withstand simulated gastric-acid conditions, indicating that the peptide could be used for oral delivery [82]. Finally, we recently showed that PepFects and other lipid-modified CPPs can be formulated with charge-neutral PMOs and form particles that are active for splice switching in different cellular models [84]. This has opened a new avenue for screening PMO sequences that are otherwise impermeable for cells. However, none of the tested peptide/PMO particles displayed any activity after intramuscular of IV delivery *in vivo*.

In another line of development, the group of Prof. Steven Dowdy introduced an interesting CPP-based technique for vectorizing siRNAs. In their setup, multiple Tat peptides were expressed as fusion proteins with a double-stranded RNA-binding domain (Tat-DRBD) [85]. Formed Tat-DRBD/siRNA nanocomplexes were shown to provide prominent RNAi activity in multiple cell lines. Moreover, potential for *in vivo* delivery was shown in a local intranasal delivery context, where Tat-DRBD/siRNA was able to produce significant gene silencing. In a follow-up study, Tat-DRBD/siRNA nanoparticles were exploited in an intracerebral glioblastoma model [86]. Here, mice inoculated with tumors were stereotactically treated locally with Tat-DRBD/siRNA nanocomplexes, with siRNA against the EGF-receptor (EGFR) and Akt2 oncogene, and the treatment led to a reduction in tumor size and substantially increased the survival of the treated animals. Interestingly, lack of systemic data and requirement to remove the GAGs from the transfection media points toward the instability of this delivery system and consequently Tat-DRBD further highlights that solely cationic vectors, without any hydrophobic domains/modifications, produce nanoparticles, which remain relatively unstable for efficient systemic application.

One aspect of CPP-based delivery that has been largely underexplored is modification of CPPs with shielding and/or targeting domains. However, a few interesting studies have been published along these lines. For example, Morris et al. used the amphipathic Pep-3 peptide for vectorization of chemically modified charged PNA

(HypNa-*p*PNA) [87]. Although the formed nanoparticles displayed high activity in cell culture, when administered IT to PC3 tumor-xenograft mice, Pep-3/HypNa-*p*PNA nanoparticles had limited activity following systemic delivery. To overcome this, the authors sought to further introduce PEG-modification to Pep-3 peptide. These PEG-carrying formulations provided significantly enhanced accumulation in the tumor tissue upon systemic administration and induced substantial tumor growth inhibition [87].

An alternative attempt to increase systemic *in vivo* delivery has, as aforementioned, been to utilize different targeting ligands to enhance the tissue-specific delivery. Kumar at al. in a very interesting approach used the rabies virus glycoprotein (RVG) peptide for targeted delivery across the blood–brain barrier (BBB) to reach the brain [88]. To allow for siRNA complex formation, they further modified the RVG peptide with the Arg9 (oligo-D-arginine) peptide. These resulting RVG-Arg9/siRNA formulations demonstrated excellent ability to induce gene silencing in the brain upon systemic administration, protecting mice against fatal viral encephalitis. In a subsequent study, the same group used T-cell-targeting CD7-specific single chain anti-body as a targeting ligand with Arg9 peptide [89]. In several systemic delivery studies in a humanized HIV mouse model, they showed how a cocktail of formulated siRNAs targeting the different axes of the HIV infection were capable of controlling viral replication, prevent T-cell loss associated with the HIV infection and actively suppress viremia in infected mice. In another study, Ren et al. explored a targeting approach for tumor-specific delivery [90]. In this, myristic acid-modified transportan (TP) peptide, modified with Lyp-1 tumor homing peptide (TP-Lyp1) was formulated with a siRNA targeting the ID4 oncogene and administered IV to mice bearing subcutaneous ovarian cancer xenografts. These treatments resulted in Lyp-1-dependent accumulation of siRNA to the tumors, efficient target gene silencing, concomitant tumor suppression, and significantly increased survival of the treated animals [90].

Recently, also a neat study exploiting the combination of PEGylation and targeting approach was published. Veiman et al. modified PepFect14 with PEG molecules, but additionally introduced it over a matrix metalloprotease (MMP) sensitive linker [91]. The rationale was that tumors that secrete MMPs would cleave and de-PEGylate the PepFect14/pDNA nanoparticles in close proximity to the tumors, which would facilitate their uptake specifically to this tissue. Upon systemic administration of these formulations in tumor-bearing mice, they were able to demonstrate that indeed such a strategy allows for specific transgene expression in the tumor tissue with minimal expression in other organs.

Different examples of CPP-mediated delivery of nucleic acids as a nanoparticles-based formulation and their applications are also described in Table 2.

4. Uptake pathways of CPP and nucleic acid conjugates and nanoparticles

As apparent from the name that was given to this class of peptides (cell penetrating), they were initially thought to possess the ability to directly translocate across cellular membranes. Early experiments mostly utilizing labeled CPPs and cell-fixation-based readouts led to the conclusion that CPPs directly penetrate through cellular membranes in an energy-independent manner [92–94]. A direct translocation mechanism was suggested because uptake at 4 °C and 37 °C was similar. Furthermore, it was easy to imagine direct physical interaction between the cationic residues of CPPs and the anionic phospholipids of the plasma membrane leading to direct membrane penetration. The models that were suggested for this mechanism were similar to those of the antimicrobial (AMPs) such as the pore formation model, carpet model, and the inverted micelle-mediated model, among others [94,95]. This similarity led to the discovery that some AMPs, which are also cationic in nature, can act as CPPs and can deliver various cargos into the cells, reviewed in [96]. The overlap between the mechanisms and applications of CPPs and AMPs has led some researchers to suggest that there might not be a clear demarcation line between the two classes of membrane active peptides [97].

In the early 2000s, endocytosis was introduced as a possible mechanism for CPP uptake [98]. Importantly, in this study, it was shown that cell fixation can lead to artefactual observation of cellular internalization, an observation which casted some doubts about the early conclusions of direct translocation experiments using cell fixation. Subsequently, endocytosis has been shown to be involved in the uptake of various CPPs and CPP complexes with nucleic acids including stearyl-Arg8, Tat-DRBD, and PepFects [76,80,85,99]. Clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis have been suggested for the uptake of CPPs and their cargo. Consequently, and as aforementioned, several approaches have been developed to enhance the escape of CPPs and their cargos out of the endosomes to reach their target subcellular compartments and avoid degradation in the lysosomal pathway. One example is a histidine-containing endosomolytic α-helical penetratin analog, EB1, which was shown to form complexes with siRNA and promote endosomal escape [100]. Other approaches utilized fusogenic peptides, such as the HA2-peptide or fatty acids conjugated to CPPs in order to facilitate release from endosomes [78,101].

Nevertheless, this has not ruled out the fact that some CPPs were still shown to utilize direct translocation mechanism in well-controlled studies [102]. Verdurmen et al. studied the uptake mechanism of several arginine-rich peptides and found that their direct penetration depends on a CPP-induced translocation of acid sphingomyelinase (ASMase) to the outer leaflet of the plasma membrane and ceramide formation [103]. Additionally, CADY peptide complexes with siRNA were shown to be internalized into different cell lines without colocalization with endosomal markers and remained active in the presence endocytosis inhibitors and at 4 °C [28].

Furthermore, an important role of HS proteoglycans in CPP uptake was suggested. This was first demonstrated for TAT peptide where the treatment with soluble heparin or with glycosaminoglycan lyases, which degrade HS chains, competitively inhibited the uptake of TAT-GFP conjugate [104]. This was also shown for several polyarginines and polylysines, where uptake was reduced in Chinese hamster ovary mutant lines deficient in either heparan sulfate or glycosaminoglycan synthesis [105]. These results led to the hypothesis that HS proteoglycans, being anionic in nature, contribute to the binding of cationic charges CPPs to the cell surface and their subsequent uptake either by direct translocation or endocytosis [106,107].

Most of the mechanisms mentioned earlier are dependent on an interaction between the cationic residues of the CPP and anionic

Table 2
Examples of CPP-mediated delivery of nucleic acids via nanoparticle-based formulations.

Peptide	Cargo	Biological effect	Reference
MPG-8, Chol-MPG8	siRNA	Efficient cyclin B1 silencing, tumor reduction and overall increased survival of the tumor-bearing mice	[72]
PepFect6	siRNA	Potent and long-lasting RNAi activity upon systemic administration in two *in vivo* models	[80]
PepFect14	SSO	Efficient splice-switching activity in the splice reporter and *in vitro* DMD model	[81]
Pep3, PEG-Pep3	asON	Effect of PEGylation in making system compatible with systemic administration and in inducing anti-tumor activity in tumor-xenograft mice	[87]
Mysistoyl-TP-Lyp1	siRNA	Demonstration of targeted delivery to the tumors, efficient tumor reduction, and increased survival of the tumor-bearing mice	[90]
PEG-MMP-PepFect14	pDNA	Tumor-specific delivery and transgene expression in the tumor-xenograft model in mice	[91]

components of the plasma membrane, either the phospholipids or HS proteoglycans, triggering uptake by direct penetration or endocytosis. Recent work in the lab of Prof. Ülo Langel has explored a different aspect of CPP–cell interaction based on the fact that with certain cargos and under certain conditions the net charge (zeta potential) of the CPP/CPP-complex is negative and not positive. It was found that the zeta potential of PepFect nanocomplexes is positive only at high peptide:nucleic acid ratios and when measured in water. However, when measured in isotonic media containing ions or serum-free media, the net charge of these complexes is predominantly negative [108]. Zeta potential is a function of the ion concentration and the pH of the medium, and it changes due to the adherence of a layer of counter ions on the surface of the nanostructure which is called the Stern layer [109]. Since the conditions where ions are present are more relevant to the biological conditions, the negatively charged surface is more likely to be the actual interface of interaction with the cells. This led to the assumption that a receptor might be involved in the uptake as the direct interaction of the newly formed anionic surface with the negatively charged plasma membrane or HS proteoglycans would lead to electrostatic repulsion. Using competitive inhibitors, siRNA knockdown and colocalization experiments, it was found that class A scavenger receptors, SCARA3 and SCARA5, are involved in the uptake of these nanocomplexes [108]. This class of innate immune receptors is known to bind negatively charged particulate ligands [110]. Other scavenger-related receptors, such as lipoprotein receptor-related protein (LRP), have been previously shown to be involved in the uptake of Tat peptide [111], and recently several studies have demonstrated the involvement of different classes of scavenger or scavenger-related receptors such as SCARAs, scavenger receptor cysteine-rich type I (M160) and SR-BI in the uptake of CPPs with different cargos including NicFects, CADY, and even with cationic polymers such as PEI [112–115]. Interestingly, an antimicrobial peptide (K8 L9) was also shown to taken up into the cells via neuropilin-1 and low-density lipoprotein-related protein receptor 1 (LRP1) at subcytotoxic concentrations [116], demonstrating even further overlap between the two classes of molecules (CPPs and AMPs).

For the conjugates, classical CPP uptake mechanisms have always been inferred. However, we have recently shown that due to their amphipathic nature, PPMOs can self-assemble into micellar structures that are then taken up via receptor-mediated endocytosis [117]. Additionally, the net charge of the conjugates was dependent on the concentration. In gel electrophoresis experiments, the migration of the conjugates was reversed between high and low concentrations displaying a net negative charge at the low concentrations and a net positive charge at high concentrations. We speculated that this was due to the change in the balance between the ions in the medium and the charges on the conjugates enabling better shielding with counterions at lower concentrations [117]. Furthermore, self-assembly into nanoparticles was also proposed as mechanism of uptake for PNA-Oligo(bicycloguanidinium) conjugates [118] and the propensity to form particles was demonstrated to enhance cellular uptake even for newly developed negatively charged ON chemistry, tcDNA [57].

Different mechanisms have been proposed for CPP conjugates and nanoparticles and distinct pathways have been postulated from direct penetration to receptor-mediated endocytosis. We would like to speculate that the apparent discrepancies might arise from different aspects of uptake of CPPs. Recent research has demonstrated that the demarcation line between conjugates and nanocomplexes is not very clear, since both PPMO and PPNA conjugates were shown to form nanoparticles [117,118]. The formation of nanoparticles might be important for the uptake based on the particle-wrapping model of endocytosis, where certain threshold of particle size should be reached for optimal uptake [119]. Below this threshold uptake will be hindered by the high energy cost required for a high curvature membrane to wrap the particle, and above it the uptake will be limited by the number of the receptors available for wrapping (Fig. 2A) [120]. This can be an explanation of the high activity of the conjugates in different body organs that are difficult to target with other nanoparticle delivery systems, which are usually restricted to the organs which have large capillary fenestrations (such as liver). The process of self-assembly, being dynamic, might be spontaneously occurring *in vivo* so that the conjugate can distribute as single molecules through smaller fenestrations into different organs (such as muscles in DMD models) and then form particles (micelles) upon accumulation and increased local concentration (Fig. 2A).

Regarding the charge, it has been shown that it is dependent on the medium and concentration, which means that different mechanisms might be dependent on different experimental settings. At low concentrations and in the presence of sufficient amount of counterions, net negative charge is predominant leading to endocytic pathways and receptor-mediated interactions. However, at higher concentrations, where the net charge is positive, direct penetration and HS proteoglygan interaction is more likely to take place (Fig. 2B). This might explain the dose-dependent toxicity of CPPs and also the overlap between CPPs and AMPs in terms of direct membrane interaction as a function of concentration. A good example of this phenomenon is the SV13-PV peptide, which has been shown to enter the cell via direct penetration at high concentrations and via endocytosis at lower concentrations in the same study [121]. Interestingly, SV13-PV was also shown to form spherical nanoparticle-like structures prior cell entry [122].

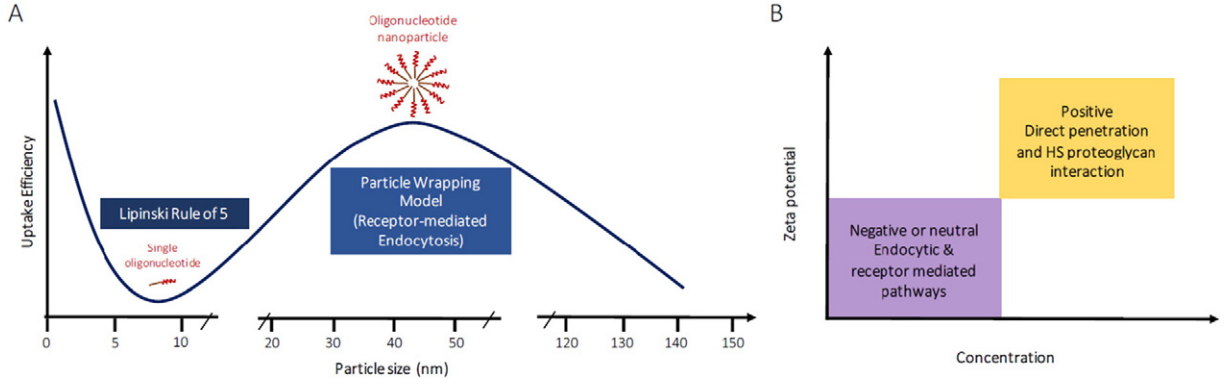

Fig. 2. *Illustrations describing the relationship between particle size, concentration, charge and the uptake of CPPs.* (A) Small molecules follow the Lipinski rule of 5 which do not apply to naked oligonucleotides, and thus they cannot passively diffuse across cellular membranes. However, large particles follow the particle-wrapping model which requires a minimum radius for optimal uptake. This might explain the activity of CPP-oligonucleotide complexes and the conjugates which were recently also shown to spontaneously form nanoparticles. (B). A demonstration of the correlation between the concentration and charge. At low concentrations, the net charge will be negative due to the presence of sufficient amount of counterions in the medium. This would mediate receptor-mediated or other endocytic pathways. However, at higher concentrations, when the net charge is positive, direct membrane interaction or interaction with negatively charged heparan sulfate proteoglygans is expected to take place.

Another example is the K8L9, which is an AMP at high concentrations, however, penetrates the cells at subcytotoxic doses via neuropilin-1 and LRP-1 receptors [116].

To conclude, findings in recent years have shed light on different mechanisms of CPP uptake and highlighted the importance of correlating the physicochemical properties of CPPs in different experimental conditions with the uptake mechanism. This has led to deeper understanding of the different aspects of the CPP interaction with cells and would hopefully lead to the design of better CPP vectors for oligonucleotide delivery in the future.

5. Perspective and future outlook of the field

Peptide-based drug delivery systems, such as CPPs, have demonstrated excellent progress in recent times for the delivery of nucleic acids and their ON analogs. As aforementioned, there are two main ON formulation strategies with CPPs: CPP-ON covalent conjugates and CPP/ON nanoparticles. Due to the high definition and known stoichiometry, covalent conjugates, for example different PPMOs, have made a huge push forward in pre-clinical studies. Noteworthy results have been achieved for a wide range of indications, including different neuromuscular and immunological disorders, viral and bacterial infections, and more. Among the different indications, most systematic work has been carried out in the context of DMD, a model disease for splice switching therapy, where PPMOs, such as B-PMOs or different Pip-PMOs, have demonstrated greatly improved bioactivity and dystrophin rescue efficacy in a range of tissues as compared to naked PMO and 2′-OMe chemistries currently used in different phases of clinical trials for DMD. Of note, these naked ON chemistries are at the moment under evaluation by the FDA for DMD but are having trouble being approved, with 2′-OMe chemistry rejected by FDA in late 2015 and PMO potentially following the same path in spring 2016, further highlighting the lack of efficacy of naked ONs and the necessity for potent drug delivery systems. In the light of this, improved splice switching chemistries, such as Pip-PMOs, offer great potential, especially as they enable therapeutic activity in other important disease-affected tissues in addition to skeletal muscles, such as in the heart, where lack of dystrophin is considered to be one of the main causes of mortality for DMD patients.

However, despite these considerable advances with CPP-ON conjugates, e.g. PPMOs, there is still some way to go until CPPs can be considered to be close for clinical translation. The reasons for this are manifold. Reasonably potent CPP-ON platforms have emerged only recently and they are mainly based on a few relatively small compound libraries, of which some demonstrate truly high activity. Therefore, there is a lot of room for improvement and considerable future effort is needed to further explore the chemical space of CPP-ON conjugates for identifying compounds with optimal efficacy/toxicity profile. For example, the commonly used arginine-rich CPPs, while demonstrating considerable activity in many tissues, also tend to induce side effects, most prominently nephrotoxicity, which puts limits on their dosing. These side effects are also specifically attributed to the CPPs, as some naked synthetic ONs, e.g. PMO, are known to have limited side effects at considerably higher doses. Obviously, lowering the amount or replacing arginines with other sources of cationic residues, for example, with different non-natural or synthetic amino acid analogs, could be an interesting subject for future studies. Moreover, pharmaceutical formulation strategies have not been explored for CPP-ON conjugates to any great extent, which could offer many advantages, from enhancing pharmacokinetics and increasing the number of available routes of administration to enhancing the safety profile and improving pharmaceutical dosage form design. Furthermore, different modification/functionalization strategies, such as targeting approaches, have received little focus, except for a few relatively inefficient homing peptide sequences. In this context, short PEG (reversible) linkers, for example, with targeting ligands from several chemical classes, could be an interesting subject for further investigation. Another important feature, which also relates to the issue of passive/active targeting, is that CPP-ON conjugates have different biodistribution profiles as compared to nanoparticle-based formulations, and by combining this further with targeting strategies, it might be possible to tailor the tissues/organs reached with ON therapeutics.

Importantly, most studies on CPP-ON conjugates have mainly concentrated on the principal biological activity of the compounds, i.e. pharmacodynamic effects, but less is known about other aspects of their behavior in the body. Therefore, future studies should strive to define in detail the full pharmacokinetic profiles of the compounds, coupled with thorough toxicological and immunological characterization, etc. Also, except for only a handful of studies, most of the pre-clinical work have been carried out in different mouse models and limited information is available for the efficacy/safety in other species and this will be an important subject for further investigation. Therefore, great effort needs to be focused on these, at the moment, experimental technologies, before their true clinical potential can be understood.

Compared to CPP-ON conjugates, CPP-based nanoparticle platforms have been investigated less thoroughly. One reason for this is that potent CPP vectors for nanoparticle applications have emerged only recently. One clear observation is that this has been made possible by introducing a range of chemical modification to the CPPs. These modifications have increased the stability of the CPP/ON nanoparticle formulations and rendered them compatible with systemic application. Moreover, introduction of different functionalization domains to the CPPs or the CPP/ON nanoparticles has enabled considerably enhanced *in vivo* delivery efficacy. It should be noted that nanoparticles have a distinct biodistribution profile, mainly distributing to organs/tissues with good blood access and/or relatively large blood vessel fenestrations, such as liver, spleen, or lung. A specific case is also different xenograft tumors with underdeveloped vasculature and lymphatic system, where nanoparticles are known to accumulate due to the so-called enhanced permeability and retention (EPR) effect, which potentially provides a way to passively target tumors. Not surprisingly, in the context of CPP/ON nanoparticles, most *in vivo* applications have concentrated on delivery to tumors.

As with conjugates, systematic screening and exploration of the chemical space for this type of vectors have been scarce and considerable future development is to be expected. For example, it would be very interesting to see how different hydrophobic modifications, which have been shown to have great effect on the activity of CPPs, would act if they would be systematically introduced to different CPPs, e.g. using fatty acid derivatives with different hydrocarbon lengths, saturation degree, amount, and orientation, and then screened for nucleic acid delivery. Moreover, such a screening approach would benefit from being combined with varying pharmaceutical formulation strategies, including a wide variety of formulation media. Furthermore, CPP/ON nanoparticle formulation should be moved to standardized production/synthesis platforms (e.g. microfluidics platforms, Jet sprays), which would keep the mixing conditions constant and provide for better reproducibility. Furthermore, as CPP/ON nanoparticles are formed due to spontaneous physical/chemical processes, these systems are to some extent always prone for instability/aggregation. Here, methodologies that would allow to segregate/homogenize nanoparticles could be important tools to produce more defined systems, especially by using these in combination with steric stabilizers (e.g. shielding compounds such as PEGs). CPP formulations thus have a lot to learn from the advances in lipid-based delivery systems, such as LNP/lipidoids, which have gone through the above-identified development steps. Interestingly, as many CPP vectors inherently possess very high delivery potential, even without systematic optimization from a pharmaceutical technology point of view, significant improvement could be expected from such modifications. Importantly, except for a few reports, different targeting strategies have not been systemically studied in the case of CPP/ON nanoparticles and this is another interesting future research space. Conclusively, CPP/ON nanoparticles have made a promising

entrance on the nucleic acid delivery stage but require considerable research, optimization, and thorough studies in different pre-clinical models to be able to fully reach their drug delivery potential in clinical settings.

An important aspect that we have also tried to emphasize in this review is that we have limited understanding of the uptake and intracellular trafficking of nucleic acids/ON, both in their naked form and when incorporated into drug delivery systems, and this impedes our capacity to fully take advantage of and chemically program the vectors for better efficiency. Therefore, considerable effort should also be focused on exploring the basic science behind the non-viral delivery process. For example, we know that different endocytic pathways play varying roles in cellular processes and some, from a delivery perspective, are probably more favored than others and these processes are typically context and cell/tissue dependent. With growing knowledge, we should try to take advantage of novel information and use for example also intracellular targeting strategies, by incorporating modifications/ligands that would mark/define the cargo for specific routes. Another interesting aspect that has been highly overlooked and has only recently been reported for lipid-based vectors is the exocytosis of the delivered compounds. This has not been studied to our knowledge in the context of CPPs or CPP and ON conjugates/nanoparticles. The implication of such process is important both in terms of uptake and re-uptake, and even more importantly in terms of re-distribution *in vivo*.

In conclusion, CPPs have demonstrated great potential for the delivery of nucleic acids and their ON analogs, as evident from many successful studies in different pre-clinical disease models. A considerable body of work also lies ahead and it can be expected that delivery peptides, such as CPPs, will have an important role to play in improving our understanding of nucleic acid therapeutics delivery and one day in successfully delivering such therapies at the bedside.

Acknowledgments

T.L. is supported by the Estonian Research Council Grant PUTJD71 and Swedish Institute's Visby program for post-doctoral scientist. K.E. by the Swedish Society of Medical Research (SSMF). S.E.L.A. by the Swedish Research Council (VR-Med and EuroNanoMedII) as well as SSMF. The authors would like to thank Fiona Lee for help with the figures and C.I. Edvard Smith, Per Lundin, and Oscar P. Wiklander for critical reading of the manuscript.

References

[1] R. Kole, A.R. Krainer, S. Altman, RNA therapeutics: beyond RNA interference and antisense oligonucleotides, Nat. Rev. Drug Discov. 11 (2012) 125–140, http://dx.doi.org/10.1038/nrd3625.
[2] F. Muntoni, M.J.A. Wood, Targeting RNA to treat neuromuscular disease, Nat. Rev. Drug Discov. 10 (2011) 621–637, http://dx.doi.org/10.1038/nrd3459.
[3] C.F. Bennett, E.E. Swayze, RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform, Annu. Rev. Pharmacol. Toxicol. 50 (2010) 259–293, http://dx.doi.org/10.1146/annurev.pharmtox.010909.105654.
[4] J.K. Watts, D.R. Corey, Silencing disease genes in the laboratory and the clinic, J. Pathol. 226 (2012) 365–379, http://dx.doi.org/10.1002/path.2993.
[5] A.G. Bader, D. Brown, M. Winkler, The promise of microRNA replacement therapy, Cancer Res. 70 (2010) 7027–7030, http://dx.doi.org/10.1158/0008-5472.CAN-10-2010.
[6] K.E. Lundin, O. Gissberg, C.I.E. Smith, Oligonucleotide therapies: the past and the present, Hum. Gene Ther. 26 (2015) 475–485, http://dx.doi.org/10.1089/hum.2015.070.
[7] A.V. Wright, J.K. Nuñez, J.A. Doudna, Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering, Cell 164 (2016) 29–44, http://dx.doi.org/10.1016/j.cell.2015.12.035.
[8] D.B.T. Cox, R.J. Platt, F. Zhang, Therapeutic genome editing: prospects and challenges, Nat. Med. 21 (2015) 121–131, http://dx.doi.org/10.1038/nm.3793.
[9] K.R. Chien, L. Zangi, K.O. Lui, Synthetic chemically modified mRNA (modRNA): toward a new technology platform for cardiovascular biology and medicine, Cold Spring Harb. Perspect. Med. 5 (2015) a014035, http://dx.doi.org/10.1101/cshperspect.a014035.
[10] R.S. Geary, D. Norris, R. Yu, C.F. Bennett, Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides, Adv. Drug Deliv. Rev. (2015), http://dx.doi.org/10.1016/j.addr.2015.01.008.
[11] R.I. Juliano, X. Ming, K. Carver, B. Laing, Cellular uptake and intracellular trafficking of oligonucleotides: implications for oligonucleotide pharmacology, Nucleic Acid Ther. 24 (2014) 101–113, http://dx.doi.org/10.1089/nat.2013.0463.
[12] L. Naldini, Gene therapy returns to centre stage, Nature 526 (2015) 351–360, http://dx.doi.org/10.1038/nature15818.
[13] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat. Rev. Drug Discov. 4 (2005) 581–593.
[14] U. Lächelt, E. Wagner, Nucleic acid therapeutics using polyplexes: a journey of 50 years (and beyond), Chem. Rev. 115 (2015) 11043–11078, http://dx.doi.org/10.1021/cr5006793.
[15] T. Lehto, E. Wagner, Sequence-defined polymers for the delivery of oligonucleotides, Nanomedicine 9 (2014) 2843–2859, http://dx.doi.org/10.2217/nnm.14.166.
[16] M. Pooga, Ü. Langel, Classes of cell-penetrating peptides, Methods Mol. Biol. Clifton N.J. 1324 (2015) 3–28, http://dx.doi.org/10.1007/978-1-4939-2806-4_1.
[17] F. Heitz, M.C. Morris, G. Divita, Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics, Br. J. Pharmacol. 157 (2009) 195–206, http://dx.doi.org/10.1111/j.1476-5381.2009.00057.x.
[18] S. Futaki, H. Hirose, I. Nakase, Arginine-rich peptides: methods of translocation through biological membranes, Curr. Pharm. Des. 19 (2013) 2863–2868.
[19] T. Lehto, K. Kurrikoff, Ü. Langel, Cell-penetrating peptides for the delivery of nucleic acids, Expert Opin. Drug Deliv. 9 (2012) 823–836, http://dx.doi.org/10.1517/17425247.2012.689285.
[20] S.A. El Andaloussi, S.M. Hammond, I. Mäger, M.J.A. Wood, Use of cell-penetrating-peptides in oligonucleotide splice switching therapy, Curr. Gene Ther. 12 (2012) 161–178.
[21] P. Boisguérin, S. Deshayes, M.J. Gait, L. O'Donovan, C. Godfrey, C.A. Betts, M.J.A. Wood, B. Lebleu, Delivery of therapeutic oligonucleotides with cell penetrating peptides, Adv. Drug Deliv. Rev. 87 (2015) 52–67, http://dx.doi.org/10.1016/j.addr.2015.02.008.
[22] A. Presente, S.F. Dowdy, PTD/CPP peptide-mediated delivery of siRNAs, Curr. Pharm. Des. 19 (2013) 2943–2947.
[23] M. Hansen, K. Kilk, U. Langel, Predicting cell-penetrating peptides, Adv. Drug Deliv. Rev. 60 (2008) 572–579, http://dx.doi.org/10.1016/j.addr.2007.09.003.
[24] R. Brock, The uptake of arginine-rich cell-penetrating peptides: putting the puzzle together, Bioconjug. Chem. 25 (2014) 863–868, http://dx.doi.org/10.1021/bc500017t.
[25] H. Margus, K. Padari, M. Pooga, Insights into cell entry and intracellular trafficking of peptide and protein drugs provided by electron microscopy, Adv. Drug Deliv. Rev. 65 (2013) 1031–1038, http://dx.doi.org/10.1016/j.addr.2013.04.013.
[26] K. Cleal, L. He, P.D. Watson, A.T. Jones, Endocytosis, intracellular traffic and fate of cell penetrating peptide based conjugates and nanoparticles, Curr. Pharm. Des. 19 (2013) 2878–2894.
[27] A. El-Sayed, H. Harashima, Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis, Mol. Ther. 21 (2013) 1118–1130, http://dx.doi.org/10.1038/mt.2013.54.
[28] A. Rydström, S. Deshayes, K. Konate, L. Crombez, K. Padari, H. Boukhaddaoui, G. Aldrian, M. Pooga, G. Divita, Direct translocation as major cellular uptake for CADY self-assembling peptide-based nanoparticles, PLoS One 6 (2011) e25924, http://dx.doi.org/10.1371/journal.pone.0025924.
[29] A. El-Sayed, S. Futaki, H. Harashima, Delivery of macromolecules using arginine-rich cell-penetrating peptides: ways to overcome endosomal entrapment, AAPS J. 11 (2009) 13–22, http://dx.doi.org/10.1208/s12248-008-9071-2.
[30] G. Sahay, W. Querbes, C. Alabi, A. Eltoukhy, S. Sarkar, C. Zurenko, E. Karagiannis, K. Love, D. Chen, R. Zoncu, Y. Buganim, A. Schroeder, R. Langer, D.G. Anderson, Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling, Nat. Biotechnol. 31 (2013) 653–658, http://dx.doi.org/10.1038/nbt.2614.
[31] P. Järver, L. O'Donovan, M.J. Gait, A chemical view of oligonucleotides for exon skipping and related drug applications, Nucleic Acid Ther. 24 (2013) 37–47, http://dx.doi.org/10.1089/nat.2013.0454.
[32] P. Jarver, T. Coursindel, S.E. Andaloussi, C. Godfrey, M.J. Wood, M.J. Gait, Peptide-mediated cell and in vivo delivery of antisense oligonucleotides and siRNA, Mol. Ther.–Nucleic Acids 1 (2012) e27, http://dx.doi.org/10.1038/mtna.2012.18.
[33] S. Deshayes, M. Morris, F. Heitz, G. Divita, Delivery of proteins and nucleic acids using a non-covalent peptide-based strategy, Adv. Drug Deliv. Rev. 60 (2008) 537–547, http://dx.doi.org/10.1016/j.addr.2007.09.005.
[34] T. Lehto, K. Ezzat, U. Langel, Peptide nanoparticles for oligonucleotide delivery, Prog. Mol. Biol. Transl. Sci. 104 (2011) 397–426, http://dx.doi.org/10.1016/B978-0-12-416020-0.00010-3.
[35] P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt, Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide, Science 254 (1991) 1497–1500, http://dx.doi.org/10.1126/science.1962210.
[36] P. Sazani, F. Gemignani, S.-H. Kang, M.A. Maier, M. Manoharan, M. Persmark, D. Bortner, R. Kole, Systemically delivered antisense oligomers upregulate gene expression in mouse tissues, Nat. Biotechnol. 20 (2002) 1228–1233, http://dx.doi.org/10.1038/nbt759.
[37] N. Bendifallah, F.W. Rasmussen, V. Zachar, P. Ebbesen, P.E. Nielsen, U. Koppelhus, Evaluation of cell-penetrating peptides (CPPs) as vehicles for intracellular delivery of antisense peptide nucleic acid (PNA), Bioconjug. Chem. 17 (2006) 750–758, http://dx.doi.org/10.1021/bc050283q.
[38] P. Lundin, H. Johansson, P. Guterstam, T. Holm, M. Hansen, U. Langel, S.E.L. Andaloussi, Distinct uptake routes of cell-penetrating peptide conjugates, Bioconjug. Chem. 19 (2008) 2535–2542, http://dx.doi.org/10.1021/bc800212j.
[39] S. El Andaloussi, P. Guterstam, U. Langel, Assessing the delivery efficacy and internalization route of cell-penetrating peptides, Nat. Protoc. 2 (2007) 2043–2047, http://dx.doi.org/10.1038/nprot.2007.302.
[40] L.C. Boffa, G. Cutrona, M. Cilli, S. Matis, G. Damonte, M.R. Mariani, E. Millo, M. Moroni, S. Roncella, F. Fedeli, M. Ferrarini, Inhibition of Burkitt's lymphoma cells

growth in SCID mice by a PNA specific for a regulatory sequence of the translocated c-myc, Cancer Gene Ther. 14 (2007) 220–226, http://dx.doi.org/10.1038/sj.cgt.7701002.
[41] F.A. Rogers, S.S. Lin, D.C. Hegan, D.S. Krause, P.M. Glazer, Targeted gene modification of hematopoietic progenitor cells in mice following systemic administration of a PNA-peptide conjugate, Mol. Ther. J. Am. Soc. Gene Ther. 20 (2012) 109–118, http://dx.doi.org/10.1038/mt.2011.163.
[42] E. Brognara, E. Fabbri, F. Aimi, A. Manicardi, N. Bianchi, A. Finotti, G. Breveglieri, M. Borgatti, R. Corradini, R. Marchelli, R. Gambari, Peptide nucleic acids targeting miR-221 modulate p27Kip1 expression in breast cancer MDA-MB-231 cells, Int. J. Oncol. 41 (2012) 2119–2127, http://dx.doi.org/10.3892/ijo.2012.1632.
[43] M.M. Fabani, C. Abreu-Goodger, D. Williams, P.A. Lyons, A.G. Torres, K.G.C. Smith, A.J. Enright, M.J. Gait, E. Vigorito, Efficient inhibition of miR-155 function in vivo by peptide nucleic acids, Nucleic Acids Res. 38 (2010) 4466–4475, http://dx.doi.org/10.1093/nar/gkq160.
[44] S. Abes, H.M. Moulton, P. Clair, P. Prevot, D.S. Youngblood, R.P. Wu, P.L. Iversen, B. Lebleu, Vectorization of morpholino oligomers by the (R-Ahx-R)4 peptide allows efficient splicing correction in the absence of endosomolytic agents, J. Control. Release 116 (2006) 304–313, http://dx.doi.org/10.1016/j.jconrel.2006.09.011.
[45] R. Abes, H.M. Moulton, P. Clair, S.T. Yang, S. Abes, K. Melikov, P. Prevot, D.S. Youngblood, P.L. Iversen, L.V. Chernomordik, B. Lebleu, Delivery of steric block morpholino oligomers by (R-X-R)4 peptides: structure–activity studies, Nucleic Acids Res. 36 (2008) 6343–6354, http://dx.doi.org/10.1093/nar/gkn541.
[46] J. Finsterer, C. Stöllberger, The heart in human dystrophinopathies, Cardiology 99 (2003) 1–19 (doi:68446).
[47] T.L.E. van Westering, C.A. Betts, M.J.A. Wood, Current understanding of molecular pathology and treatment of cardiomyopathy in duchenne muscular dystrophy, Mol. Basel Switz. 20 (2015) 8823–8855, http://dx.doi.org/10.3390/molecules20058823.
[48] A.G.L. Douglas, M.J.A. Wood, Splicing therapy for neuromuscular disease, Mol. Cell. Neurosci. 56 (2013) 169–185, http://dx.doi.org/10.1016/j.mcn.2013.04.005.
[49] T. Koo, M.J. Wood, Clinical trials using antisense oligonucleotides in duchenne muscular dystrophy, Hum. Gene Ther. 24 (2013) 479–488, http://dx.doi.org/10.1089/hum.2012.234.
[50] H. Yin, H.M. Moulton, Y. Seow, C. Boyd, J. Boutilier, P. Iverson, M.J. Wood, Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function, Hum. Mol. Genet. 17 (2008) 3909–3918, http://dx.doi.org/10.1093/hmg/ddn293.
[51] B. Wu, H.M. Moulton, P.L. Iversen, J. Jiang, J. Li, J. Li, C.F. Spurney, A. Sali, A.D. Guerron, K. Nagaraju, T. Doran, P. Lu, X. Xiao, Q.L. Lu, Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14814–14819, http://dx.doi.org/10.1073/pnas.0805676105.
[52] H. Yin, H.M. Moulton, C. Betts, Y. Seow, J. Boutilier, P.L. Iverson, M.J.A. Wood, A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice, Hum. Mol. Genet. 18 (2009) 4405–4414, http://dx.doi.org/10.1093/hmg/ddp395.
[53] H. Yin, A.F. Saleh, C. Betts, P. Camelliti, Y. Seow, S. Ashraf, A. Arzumanov, S. Hammond, T. Merritt, M.J. Gait, M.J. Wood, Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice, Mol. Ther. 19 (2011) 1295–1303, http://dx.doi.org/10.1038/mt.2011.79.
[54] C. Betts, A.F. Saleh, A.A. Arzumanov, S.M. Hammond, C. Godfrey, T. Coursindel, M.J. Gait, M.J. Wood, Pip6-PMO, a new generation of peptide-oligonucleotide conjugates with improved cardiac exon skipping activity for DMD treatment, Mol. Ther.–Nucleic Acids 1 (2012) e38, http://dx.doi.org/10.1038/mtna.2012.30.
[55] C.A. Betts, A.F. Saleh, C.A. Carr, S.M. Hammond, A.M.L. Coenen-Stass, C. Godfrey, G. McClorey, M.A. Varela, T.C. Roberts, K. Clarke, M.J. Gait, M.J.A. Wood, Prevention of exercised induced cardiomyopathy following Pip-PMO treatment in dystrophic mdx mice, Sci. Rep. 5 (2015), http://dx.doi.org/10.1038/srep08986.
[56] C. Godfrey, S. Muses, G. McClorey, K.E. Wells, T. Coursindel, R.L. Terry, C. Betts, S. Hammond, L. O'Donovan, J. Hildyard, S.E. Andaloussi, M.J. Gait, M.J. Wood, D.J. Wells, How much dystrophin is enough: the physiological consequences of different levels of dystrophin in the mdx mouse, Hum. Mol. Genet. 24 (2015) 4225–4237, http://dx.doi.org/10.1093/hmg/ddv155.
[57] A. Goyenvalle, G. Griffith, A. Babbs, S.E. Andaloussi, K. Ezzat, A. Avril, B. Dugovic, R. Chaussenot, A. Ferry, T. Voit, H. Amthor, C. Bühr, S. Schürch, M.J.A. Wood, K.E. Davies, C. Vaillend, C. Leumann, L. Garcia, Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers, Nat. Med. (2015), http://dx.doi.org/10.1038/nm.3765 (advance online publication).
[58] A.J. Leger, L.M. Mosquea, N.P. Clayton, I.-H. Wu, T. Weeden, C.A. Nelson, L. Phillips, E. Roberts, P.A. Piepenhagen, S.H. Cheng, B.M. Wentworth, Systemic delivery of a peptide-linked morpholino oligonucleotide neutralizes mutant RNA toxicity in a mouse model of myotonic dystrophy, Nucleic Acid Ther. 23 (2013) 109–117, http://dx.doi.org/10.1089/nat.2012.0404.
[59] B. Bestas, P.M.D. Moreno, K.E.M. Blomberg, D.K. Mohammad, A.F. Saleh, T. Sutlu, J.Z. Nordin, P. Guterstam, M.O. Gustafsson, S. Kharazi, B. Piątosa, T.C. Roberts, M.A. Behlke, M.J.A. Wood, M.J. Gait, K.E. Lundin, S. El Andaloussi, R. Månsson, A. Berglöf, J. Wengel, C.I.E. Smith, Splice-correcting oligonucleotides restore BTK function in X-linked agammaglobulinemia model, J. Clin. Invest. 124 (2014) 4067–4081, http://dx.doi.org/10.1172/JCI76175.
[60] S.-H. Lai, D.A. Stein, A. Guerrero-Plata, S.-L. Liao, T. Ivanciuc, C. Hong, P.L. Iversen, A. Casola, R.P. Garofalo, Inhibition of respiratory syncytial virus infections with morpholino oligomers in cell cultures and in mice, Mol. Ther. J. Am. Soc. Gene Ther. 16 (2008) 1120–1128, http://dx.doi.org/10.1038/mt.2008.81.
[61] T. Opriessnig, D. Patel, R. Wang, P.G. Halbur, X.-J. Meng, D.A. Stein, Y.-J. Zhang, Inhibition of porcine reproductive and respiratory syndrome virus infection in piglets by a peptide-conjugated morpholino oligomer, Antivir. Res. 91 (2011) 36–42, http://dx.doi.org/10.1016/j.antiviral.2011.04.012.
[62] R. Burrer, B.W. Neuman, J.P.C. Ting, D.A. Stein, H.M. Moulton, P.L. Iversen, P. Kuhn, M.J. Buchmeier, Antiviral effects of antisense morpholino oligomers in murine coronavirus infection models, J. Virol. 81 (2007) 5637–5648, http://dx.doi.org/10.1128/JVI.02360-06.
[63] D.A. Stein, C.Y.-H. Huang, S. Silengo, A. Amantana, S. Crumley, R.E. Blouch, P.L. Iversen, R.M. Kinney, Treatment of AG129 mice with antisense morpholino oligomers increases survival time following challenge with dengue 2 virus, J. Antimicrob. Chemother. 62 (2008) 555–565, http://dx.doi.org/10.1093/jac/dkn221.
[64] T.S. Deas, C.J. Bennett, S.A. Jones, M. Tilgner, P. Ren, M.J. Behr, D.A. Stein, P.L. Iversen, L.D. Kramer, K.A. Bernard, P.-Y. Shi, In vitro resistance selection and in vivo efficacy of morpholino oligomers against West Nile virus, Antimicrob. Agents Chemother. 51 (2007) 2470–2482, http://dx.doi.org/10.1128/AAC.00069-07.
[65] L.D. Tilley, B.L. Mellbye, S.E. Puckett, P.L. Iversen, B.L. Geller, Antisense peptide-phosphorodiamidate morpholino oligomer conjugate: dose–response in mice infected with *Escherichia coli*, J. Antimicrob. Chemother. 59 (2007) 66–73, http://dx.doi.org/10.1093/jac/dkl444.
[66] D. Wesolowski, H.S. Tae, N. Gandotra, P. Llopis, N. Shen, S. Altman, Basic peptide-morpholino oligomer conjugate that is very effective in killing bacteria by gene-specific and nonspecific modes, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 16582–16587, http://dx.doi.org/10.1073/pnas.1112561108.
[67] R.G. Panchal, B.L. Geller, B. Mellbye, D. Lane, P.L. Iversen, S. Bavari, Peptide conjugated phosphorodiamidate morpholino oligomers increase survival of mice challenged with Ames Bacillus anthracis, Nucleic Acid Ther. 22 (2012) 316–322, http://dx.doi.org/10.1089/nat.2012.0362.
[68] B.L. Geller, K. Marshall-Batty, F.J. Schnell, M.M. McKnight, P.L. Iversen, D.E. Greenberg, Gene-silencing antisense oligomers inhibit acinetobacter growth in vitro and in vivo, J. Infect. Dis. 208 (2013) 1553–1560, http://dx.doi.org/10.1093/infdis/jit460.
[69] M.C. Morris, P. Vidal, L. Chaloin, F. Heitz, G. Divita, A new peptide vector for efficient delivery of oligonucleotides into mammalian cells, Nucleic Acids Res. 25 (1997) 2730–2736.
[70] T.B. Wyman, F. Nicol, O. Zelphati, P.V. Scaria, C. Plank, F.C. Szoka, Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers, Biochemistry (Mosc) 36 (1997) 3008–3017, http://dx.doi.org/10.1021/bi9618474.
[71] M.C. Morris, L. Chaloin, J. Méry, F. Heitz, G. Divita, A novel potent strategy for gene delivery using a single peptide vector as a carrier, Nucleic Acids Res. 27 (1999) 3510–3517.
[72] L. Crombez, M.C. Morris, S. Dufort, G. Aldrian-Herrada, Q. Nguyen, G. Mc Master, J.L. Coll, F. Heitz, G. Divita, Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth, Nucleic Acids Res. 37 (2009) 4559–4569, http://dx.doi.org/10.1093/nar/gkp451.
[73] L. Crombez, G. Aldrian-Herrada, K. Konate, Q.N. Nguyen, G.K. McMaster, R. Brasseur, F. Heitz, G. Divita, A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells, Mol. Ther. 17 (2009) 95–103, http://dx.doi.org/10.1038/mt.2008.215.
[74] E. Eiríksdóttir, K. Konate, Ü. Langel, G. Divita, S. Deshayes, Secondary structure of cell-penetrating peptides controls membrane interaction and insertion, Biochim. Biophys. Acta Biomembr. 1798 (2010) 1119–1128, http://dx.doi.org/10.1016/j.bbamem.2010.03.005.
[75] Y.-W. Kim, T.N. Grossmann, G.L. Verdine, Synthesis of all-hydrocarbon stapled α-helical peptides by ring-closing olefin metathesis, Nat. Protoc. 6 (2011) 761–771, http://dx.doi.org/10.1038/nprot.2011.324.
[76] T. Lehto, R. Abes, N. Oskolkov, J. Suhorutsenko, D.M. Copolovici, I. Mager, J.R. Viola, O.E. Simonson, K. Ezzat, P. Guterstam, E. Eriste, C.I. Smith, B. Lebleu, A. Samir El, U. Langel, Delivery of nucleic acids with a stearylated (RxR)4 peptide using a non-covalent co-incubation strategy, J. Control. Release 141 (2010) 42–51, http://dx.doi.org/10.1016/j.jconrel.2009.08.028.
[77] Y. Sato, T. Nakamura, Y. Yamada, H. Akita, H. Harashima, Chapter six – multifunctional enveloped nanodevices (MENDs), in: Leaf Huang, Dexi Liu, Ernst Wagner (Eds.), Adv. Genet. Academic Press 2014, pp. 139–204 http://www.sciencedirect.com/science/article/pii/B9780128001486000067 (accessed February 23, 2016).
[78] M. Mäe, S. El Andaloussi, P. Lundin, N. Oskolkov, H.J. Johansson, P. Guterstam, U. Langel, A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy, J. Control. Release Off. J. Control. Release Soc. 134 (2009) 221–227, http://dx.doi.org/10.1016/j.jconrel.2008.11.025.
[79] T. Lehto, O.E. Simonson, I. Mäger, K. Ezzat, H. Sork, D.-M. Copolovici, J.R. Viola, E.M. Zaghloul, P. Lundin, P.M. Moreno, M. Mäe, N. Oskolkov, J. Suhorutšenko, C.E. Smith, S.E. Andaloussi, A peptide-based vector for efficient gene transfer in vitro and in vivo, Mol. Ther. 19 (2011) 1457–1467, http://dx.doi.org/10.1038/mt.2011.10.
[80] S.E. Andaloussi, T. Lehto, I. Mager, K. Rosenthal-Aizman, I. Oprea, O.E. Simonson, H. Sork, K. Ezzat, D.M. Copolovici, K. Kurrikoff, J.R. Viola, E.M. Zaghloul, R. Sillard, H.J. Johansson, F. Said Hassane, P. Guterstam, J. Suhorutsenko, P.M. Moreno, N. Oskolkov, J. Halldin, U. Tedebark, A. Metspalu, B. Lebleu, J. Lehtio, C.I. Smith, U. Langel, Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo, Nucleic Acids Res. 39 (2011) 3972–3987, http://dx.doi.org/10.1093/nar/gkq1299.
[81] K. Ezzat, S.E. Andaloussi, E.M. Zaghloul, T. Lehto, S. Lindberg, P.M. Moreno, J.R. Viola, T. Magdy, R. Abdo, P. Guterstam, R. Sillard, S.M. Hammond, M.J. Wood, A.A. Arzumanov, M.J. Gait, C.I. Smith, M. Hallbrink, U. Langel, PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation, Nucleic Acids Res. 39 (2011) 5284–5298, http://dx.doi.org/10.1093/nar/gkr072.

[82] K. Ezzat, E.M. Zaghloul, S. El Andaloussi, T. Lehto, R. El-Sayed, T. Magdy, C.I. Smith, U. Langel, Solid formulation of cell-penetrating peptide nanocomplexes with siRNA and their stability in simulated gastric conditions, J. Control. Release 162 (2012) 1–8, http://dx.doi.org/10.1016/j.jconrel.2012.06.006.
[83] K.-L. Veiman, I. Mäger, K. Ezzat, H. Margus, T. Lehto, K. Langel, K. Kurrikoff, P. Arukuusk, J. Suhorutšenko, K. Padari, M. Pooga, T. Lehto, Ü. Langel, PepFect14 peptide vector for efficient gene delivery in cell cultures, Mol. Pharm. 10 (2013) 199–210, http://dx.doi.org/10.1021/mp3003557.
[84] P. Järver, E.M. Zaghloul, A.A. Arzumanov, A.F. Saleh, G. McClorey, S.M. Hammond, M. Hällbrink, Ü. Langel, C.I.E. Smith, M.J.A. Wood, M.J. Gait, S.E.L. Andaloussi, Peptide nanoparticle delivery of charge-neutral splice-switching morpholino oligonucleotides, Nucleic Acid Ther. 25 (2015) 65–77, http://dx.doi.org/10.1089/nat.2014.0511.
[85] A. Eguchi, B.R. Meade, Y.C. Chang, C.T. Fredrickson, K. Willert, N. Puri, S.F. Dowdy, Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein, Nat. Biotechnol. 27 (2009) 567–571, http://dx.doi.org/10.1038/nbt.1541.
[86] H. Michiue, A. Eguchi, M. Scadeng, S.F. Dowdy, Induction of in vivo synthetic lethal RNAi responses to treat glioblastoma, Cancer Biol. Ther. 8 (2009) 2306–2313.
[87] M.C. Morris, E. Gros, G. Aldrian-Herrada, M. Choob, J. Archdeacon, F. Heitz, G. Divita, A non-covalent peptide-based carrier for in vivo delivery of DNA mimics, Nucleic Acids Res. 35 (2007) e49, http://dx.doi.org/10.1093/nar/gkm053.
[88] P. Kumar, H. Wu, J.L. McBride, K.-E. Jung, M. Hee Kim, B.L. Davidson, S. Kyung Lee, P. Shankar, N. Manjunath, Transvascular delivery of small interfering RNA to the central nervous system, Nature 448 (2007) 39–43, http://dx.doi.org/10.1038/nature05901.
[89] P. Kumar, H.-S. Ban, S.-S. Kim, H. Wu, T. Pearson, D.L. Greiner, A. Laouar, J. Yao, V. Haridas, K. Habiro, Y.-G. Yang, J.-H. Jeong, K.-Y. Lee, Y.-H. Kim, S.W. Kim, M. Peipp, G.H. Fey, N. Manjunath, L.D. Shultz, S.-K. Lee, P. Shankar, T Cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice, Cell 134 (2008) 577–586, http://dx.doi.org/10.1016/j.cell.2008.06.034.
[90] Y. Ren, H.W. Cheung, G. von Maltzhan, A. Agrawal, G.S. Cowley, B.A. Weir, J.S. Boehm, P. Tamayo, A.M. Karst, J.F. Liu, M.S. Hirsch, J.P. Mesirov, R. Drapkin, D.E. Root, J. Lo, V. Fogal, E. Ruoslahti, W.C. Hahn, S.N. Bhatia, Targeted tumor-penetrating siRNA nanocomplexes for credentialing the ovarian cancer oncogene ID4, Sci. Transl. Med. 4 (2012) 147ra112, http://dx.doi.org/10.1126/scitranslmed.3003778.
[91] K.-L. Veiman, K. Künnapuu, T. Lehto, K. Kiisholts, K. Pärn, Ü. Langel, K. Kurrikoff, PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo, J. Control. Release 209 (2015) 238–247, http://dx.doi.org/10.1016/j.jconrel.2015.04.038.
[92] E. Vivès, P. Brodin, B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, J. Biol. Chem. 272 (1997) 16010–16017.
[93] D. Derossi, A.H. Joliot, G. Chassaing, A. Prochiantz, The third helix of the Antennapedia homeodomain translocates through biological membranes, J. Biol. Chem. 269 (1994) 10444–10450.
[94] D. Derossi, S. Calvet, A. Trembleau, A. Brunissen, G. Chassaing, A. Prochiantz, Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent, J. Biol. Chem. 271 (1996) 18188–18193.
[95] Z. Oren, Y. Shai, Mode of action of linear amphipathic alpha-helical antimicrobial peptides, Biopolymers 47 (1998) 451–463, http://dx.doi.org/10.1002/(SICI)1097-0282(1998)47:6<451::AID-BIP4>3.0.CO;2-F.
[96] K. Splith, I. Neundorf, Antimicrobial peptides with cell-penetrating peptide properties and vice versa, Eur. Biophys. J. EBJ 40 (2011) 387–397, http://dx.doi.org/10.1007/s00249-011-0682-7.
[97] S.T. Henriques, M.N. Melo, M.A.R.B. Castanho, Cell-penetrating peptides and antimicrobial peptides: how different are they? Biochem. J. 399 (2006) 1–7, http://dx.doi.org/10.1042/BJ20061100.
[98] J.P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides. a reevaluation of the mechanism of cellular uptake, J. Biol. Chem. 278 (2003) 585–590, http://dx.doi.org/10.1074/jbc.M209548200.
[99] I.A. Khalil, S. Futaki, M. Niwa, Y. Baba, N. Kaji, H. Kamiya, H. Harashima, Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation, Gene Ther. 11 (2004) 636–644, http://dx.doi.org/10.1038/sj.gt.3302128.
[100] P. Lundberg, S. El-Andaloussi, T. Sütlü, H. Johansson, U. Langel, Delivery of short interfering RNA using endosomolytic cell-penetrating peptides, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 21 (2007) 2664–2671, http://dx.doi.org/10.1096/fj.06-6502com.
[101] A. El-Sayed, T. Masuda, I. Khalil, H. Akita, H. Harashima, Enhanced gene expression by a novel stearylated INF7 peptide derivative through fusion independent endosomal escape, J. Control. Release Off. J. Control. Release Soc. 138 (2009) 160–167, http://dx.doi.org/10.1016/j.jconrel.2009.05.018.
[102] C. Palm-Apergi, A. Lorents, K. Padari, M. Pooga, M. Hällbrink, The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 23 (2009) 214–223, http://dx.doi.org/10.1096/fj.08-110254.
[103] W.P.R. Verdurmen, M. Thanos, I.R. Ruttekolk, E. Gulbins, R. Brock, Cationic cell-penetrating peptides induce ceramide formation via acid sphingomyelinase: implications for uptake, J. Control. Release Off. J. Control. Release Soc. 147 (2010) 171–179, http://dx.doi.org/10.1016/j.jconrel.2010.06.030.
[104] M. Tyagi, M. Rusnati, M. Presta, M. Giacca, Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans, J. Biol. Chem. 276 (2001) 3254–3261, http://dx.doi.org/10.1074/jbc.M006701200.
[105] J.C. Mai, H. Shen, S.C. Watkins, T. Cheng, P.D. Robbins, Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate, J. Biol. Chem. 277 (2002) 30208–30218, http://dx.doi.org/10.1074/jbc.M204202200.
[106] M. Belting, Heparan sulfate proteoglycan as a plasma membrane carrier, Trends Biochem. Sci. 28 (2003) 145–151, http://dx.doi.org/10.1016/S0968-0004(03)00031-8.
[107] G.M.K. Poon, J. Gariépy, Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells, Biochem. Soc. Trans. 35 (2007) 788–793, http://dx.doi.org/10.1042/BST0350788.
[108] K. Ezzat, H. Helmfors, O. Tudoran, C. Juks, S. Lindberg, K. Padari, S. El-Andaloussi, M. Pooga, U. Langel, Scavenger receptor-mediated uptake of cell-penetrating peptide nanocomplexes with oligonucleotides, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 26 (2012) 1172–1180, http://dx.doi.org/10.1096/fj.11-191536.
[109] M.M. Domingues, P.S. Santiago, M.A.R.B. Castanho, N.C. Santos, What can light scattering spectroscopy do for membrane-active peptide studies? J. Pept. Sci. Off. Publ. Eur. Pept. Soc. 14 (2008) 394–400, http://dx.doi.org/10.1002/psc.1007.
[110] J. Canton, D. Neculai, S. Grinstein, Scavenger receptors in homeostasis and immunity, Nat. Rev. Immunol. 13 (2013) 621–634, http://dx.doi.org/10.1038/nri3515.
[111] Y. Liu, M. Jones, C.M. Hingtgen, G. Bu, N. Laribee, R.E. Tanzi, R.D. Moir, A. Nath, J.J. He, Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands, Nat. Med. 6 (2000) 1380–1387, http://dx.doi.org/10.1038/82199.
[112] E. Kondo, K. Saito, Y. Tashiro, K. Kamide, S. Uno, T. Furuya, M. Mashita, K. Nakajima, T. Tsumuraya, N. Kobayashi, M. Nishibori, M. Tanimoto, M. Matsushita, Tumour lineage-homing cell-penetrating peptides as anticancer molecular delivery systems, Nat. Commun. 3 (2012) 951, http://dx.doi.org/10.1038/ncomms1952.
[113] S. Lindberg, J. Regberg, J. Eriksson, H. Helmfors, A. Muñoz-Alarcón, A. Srimanee, R.A. Figueroa, E. Hallberg, K. Ezzat, Ü. Langel, A convergent uptake route for peptide- and polymer-based nucleotide delivery systems, J. Control. Release 206 (2015) 58–66, http://dx.doi.org/10.1016/j.jconrel.2015.03.009.
[114] A. Srimanee, J. Regberg, M. Hällbrink, O. Vajragupta, Ü. Langel, Role of scavenger receptors in peptide-based delivery of plasmid DNA across a blood–brain barrier model, Int. J. Pharm. 500 (2016) 128–135, http://dx.doi.org/10.1016/j.ijpharm.2016.01.014.
[115] P. Arukuusk, L. Pärnaste, H. Margus, N.K.J. Eriksson, L. Vasconcelos, K. Padari, M. Pooga, U. Langel, Differential endosomal pathways for radically modified peptide vectors, Bioconjug. Chem. 24 (2013) 1721–1732, http://dx.doi.org/10.1021/bc4002757.
[116] M. Kohno, T. Horibe, K. Ohara, S. Ito, K. Kawakami, The membrane-lytic peptides K8 L9 and melittin enter cancer cells via receptor endocytosis following subcytotoxic exposure, Chem. Biol. 21 (2014) 1522–1532, http://dx.doi.org/10.1016/j.chembiol.2014.09.008.
[117] K. Ezzat, Y. Aoki, T. Koo, G. McClorey, L. Benner, A. Coenen-Stass, L. O'Donovan, T. Lehto, A. Garcia-Guerra, J. Nordin, A.F. Saleh, M. Behlke, J. Morris, A. Goyenvalle, B. Dugovic, C. Leumann, S. Gordon, M.J. Gait, S. El-Andaloussi, M.J. Wood, Self-assembly into nanoparticles is essential for receptor mediated uptake of therapeutic antisense oligonucleotides, Nano Lett. 15 (2015) 4364–4373, http://dx.doi.org/10.1021/acs.nanolett.5b00490.
[118] J. Valero, T. Shiraishi, J. de Mendoza, P.E. Nielsen, Cellular antisense activity of PNA-Oligo(bicycloguanidinium) conjugates forming self-assembled nanoaggregates, Chembiochem Eur. J. Chem. Biol. 16 (2015) 1593–1600, http://dx.doi.org/10.1002/cbic.201500172.
[119] H. Gao, W. Shi, L.B. Freund, Mechanics of receptor-mediated endocytosis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 9469–9474, http://dx.doi.org/10.1073/pnas.0503879102.
[120] W. Shi, J. Wang, X. Fan, H. Gao, Size and shape effects on diffusion and absorption of colloidal particles near a partially absorbing sphere: implications for uptake of nanoparticles in animal cells, Phys. Rev. E Stat. Nonlinear Soft Matter Phys. 78 (2008) 061914, http://dx.doi.org/10.1103/PhysRevE.78.061914.
[121] M. Mano, C. Teodósio, A. Paiva, S. Simões, M.C. Pedroso de Lima, On the mechanisms of the internalization of S4(13)-PV cell-penetrating peptide, Biochem. J. 390 (2005) 603–612, http://dx.doi.org/10.1042/BJ20050577.
[122] K. Padari, K. Koppel, A. Lorents, M. Hällbrink, M. Mano, M.C. Pedroso de Lima, M. Pooga, S4(13)-PV cell-penetrating peptide forms nanoparticle-like structures to gain entry into cells, Bioconjug. Chem. 21 (2010) 774–783, http://dx.doi.org/10.1021/bc900577e.
[123] M. Pooga, U. Soomets, M. Hällbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.X. Hao, X.J. Xu, Z. Wiesenfeld-Hallin, T. Hökfelt, T. Bartfai, U. Langel, Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo, Nat. Biotechnol. 16 (1998) 857–861, http://dx.doi.org/10.1038/nbt0998-857.