EXPERT OPINION

Cell-penetrating peptides for the delivery of nucleic acids

Taavi Lehto†, Kaido Kurrikoff & Ülo Langel
† *University of Tartu, Institute of Technology, Laboratory of Molecular Biotechnology, Tartu, Estonia*

Introduction: Different gene therapy approaches have gained extensive interest lately and, after many initial hurdles, several promising approaches have reached to the clinics. Successful implementation of gene therapy is heavily relying on finding efficient measures to deliver genetic material to cells. Recently, non-viral delivery of nucleic acids and their analogs has gained significant interest. Among non-viral vectors, cell-penetrating peptides (CPPs) have been extensively used for the delivery of nucleic acids both *in vitro* and *in vivo.*

Areas covered: In this review we will discuss recent advances of CPP-mediated delivery of nucleic acid-based cargo, concentrating on the delivery of plasmid DNA, splice-correcting ONs, and small-interfering RNAs.

Expert opinion: CPPs have proved their potential as carriers for nucleic acids. However, similarly to other non-viral vectors, CPPs require further development, as efficient systemic delivery is still seldom achieved. To achieve this, CPPs should be modified with entities that would allow better endosomal escape, targeting of specific tissues and cells, and shielding agents that increase the half-life of the vehicles. Finally, to understand the clinical potential of CPPs, they require more thorough investigations in clinically relevant disease models and in pre-clinical and clinical studies.

Keywords: cell-penetrating peptide, gene delivery, nanoparticle, oligonucleotide delivery, siRNA delivery

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1. Introduction

Different gene therapy approaches have gained significant focus lately and, after many initial roadblocks, gene therapy is now considered as viable treatment option for different disorders, including combined immunodeficiency syndrome (SCID) and Wiscott-Aldridge syndrome [1]. The repertoire of gene therapy approaches is increasing and, additionally to classical gene therapy by the means of introduction of a therapeutic gene, different methods utilizing antisense-based approaches have become available [2,3]. These antisense-based techniques allow gene expression regulation at transcriptional or post-transcriptional level and, due to their specific nature, they are today intensely investigated in numerous clinical trials for a variety of diseases. Independent of the approach, molecules used in gene therapy settings are based on nucleic acids and their analogs, which due to their physicochemical properties and instability have very limited bioavailability. Therefore, these nucleic acids *per se* require assistance in their delivery and the lack of efficient and safe delivery vectors for genetic material has been the major limitation in the successful implementation of gene therapy.

Broadly, vectorization of nucleic acids can be achieved via two approaches – by using viral or non-viral delivery vectors. Viral vectors are the most efficient delivery vehicles for genetic materials in general and although the safety profile of the viral vectors has improved they have their limitations [4]. Importantly, viral vectors are

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Article highlights.

- Cell-penetrating peptides (CPPs) are a group of delivery vehicles that allow efficient intracellular delivery of different bioactive molecules, both in cell cultures and *in vivo*.
- CPPs can vectorize nucleic acids, including different oligonucleotides (ONs) by two approaches – via covalent conjugation or nanoparticle formation with cargo molecules.
- CPPs are efficient delivery vehicles for the delivery of plasmid DNA (pDNA), splice-correcting oligonucleotides (SCOs), and small-interfering RNAs (siRNAs).
- Chemical modifications on CPPs can have a substantial effect on their delivery properties.
- Addition of functionalizing modification – for endosomal escape, shielding and targeting – on CPPs is required for further development of CPPs, especially to achieve targeted delivery after systemic administration.
- More work is required in evaluating the CPP-based platforms in disease-relevant settings, pre-clinical and clinical studies.

This box summarizes key points contained in the article.

restricted by cargo-carrying capacity and are not compatible with the transient delivery of synthetic antisense-based oligonucleotides (ONs), for instance splice-correcting ONs (SCOs) and small-interfering RNAs (siRNAs). This has merited in a significant interest toward non-viral delivery methods – to find safer alternatives for gene delivery and develop vectors for shorter ONs. Non-viral vectors are usually based on different cationic entities, for example different lipids, synthetic polymers or peptides [5]. Among these, cationic lipids and synthetic polymers, known as lipoplexes and polyplexes, respectively, have been studied most extensively. Recently also a group of non-viral vectors, named cell-penetrating peptides (CPPs), has received much attention for their ability to transport nucleic acids into cells both *in vitro* and *in vivo* [6].

CPPs are relatively short peptides, 5 – 30 amino acids in length, they are cationic and/or amphipathic and have a net positive charge, with the ability to gain access to the cell interior by means of different mechanisms, mainly including endocytosis, and with the capacity to promote intracellular delivery of covalently or non-covalently conjugated bioactive cargo [6]. While CPPs are hard to define, the common denominator between all the CPP is that they are capable of mediating the delivery of various macromolecules across the cellular membranes, including peptides, proteins, plasmid DNA (pDNA), antisense ONs (asONs), SCOs, siRNAs, anti-microRNAs (antiMiRs), and nanoparticles both *in vitro* and *in vivo* (as reviewed in [7-11]). A selection of most broadly used CPPs are presented in **Table 1**.

Even though the delivery properties of CPPs are undoubted, the uptake mechanism of CPPs is still controversial. Broadly, there are two ways that CPPs are believed to gain access to the cell interior – by endocytic pathways or by direct penetration. It is generally accepted that CPPs exploit an energy-dependent internalization mechanism that involves interaction with cellular membrane elements, such as heparan sulfates [12] or receptors [13], and endocytosis of different types, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis, as extensively described in [8,14,15]. For some particular CPPs, cargoes and delivery conditions, direct penetration is the major route. For example, MPG and CADY peptide have been shown to internalize by non-endocytic internalization mechanism [16,17].

As CPPs utilize mainly endocytic pathways to gain access to the cells and as a result the endosomal entrapment serves as a main limiting factor in the bioavailability of CPP-mediated nucleic acid delivery. This has led to intense investigations to overcome this limitation, from the design of peptides with amphipathic properties that are more active toward the endosomal membranes to more specific modifications that would add endosomotropic properties, for instance membrane-disruptive peptides, polymers, fusogenic lipids (as recently reviewed in [18,19]). For example, a viral protein-derived fusogenic HA2 peptide is known to enable endosomal escape by adopting α-helical structure at endosomal pH and this mediates partial disruption of the endosomal membrane. Wadia *et al.* were first to utilize this peptide with CPPs and they showed that by adding this segment to the Tat peptide, the uptake of Tat-Cre fusion protein was enhanced [20]. Histidine-rich motifs have been used to augment the endosomolytical properties. For example, Lundberg *et al.* designed a penetratin analog by introducing histidine moieties to induce the formation of α-helical structure in the early/late endosomes and promote endosomal escape [21]. Similar approach has been used with Tat peptide, where significant enhancement of pDNA delivery was observed both *in vitro* and *in vivo* [22]. Histidines are believed to enable endosomal escape by being protonated at low pH, leading to subsequent swelling and rupture of the endosomes. Additionally, different endosomotropic agents have been used. For example, fusogenic lipids have been frequently added to the CPP formulations to enhance endosomal escape. Thus, dioleoylphosphatidyl-ethanolamine (DOPE) has been used as an integral part of the multifunctional envelope-type nano-device (MEND) platform to promote endosomal escape [23,24]. Recently, our group reported a novel covalent endosomolytic modification – a trifuoromethylquinoline – that greatly enhanced endosomal escape [25]. Also, modification of CPPs with fatty acids have been shown to enhance the bioavailability of associated cargo. Particularly, addition of stearic acid modification to CPPs has been shown to increase the delivery efficiency of pDNA [26,27], SCOs [28], and siRNA [29,30].

In this review, we are surveying the utilization of CPP-based non-viral delivery vectors for the delivery of nucleic acids and their analogs, concentrating on the latest advances in the CPP-mediated delivery of pDNA, SCOs, and siRNAs.

Table 1. Examples of different CPPs.

CPP	Sequence	Origin	Ref.
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	Chimeric	[95]
TP10	AGYLLGKINLKALAALAKKIL-NH_2	Chimeric	[96]
MPG	GALFLGWLGAAGSTMGAPKKKRKV-cya	Chimeric	[38]
Pep-1	KETWWETWWTEWSQPKKKRKV-cya	Chimeric	[97]
Penetratin	RQIKIWFQNRRMKWKK	Protein derived	[85]
MAP	KLALKLALKALKAALKLA-NH_2	Synthetic	[98]
CADY	GLWRALWRLLRSLWRLLWRA-cya	Synthetic	[81]
Tat (48-60)	GRKKRRQRRRPPQ	Protein derived	[99]
Oligoarginine	(R)n	Synthetic	[27]

Cya: C-terminal cysteamide.

2. Vectorization of nucleic acids with CPPs

In general, there are two main strategies of how cargo molecules could be vectorized with CPPs – via covalent or non-covalent approach. Most of the studies involving CPPs have utilized covalent conjugation method, because chemical conjugation results in a well-defined chemical entity and, if looking on the clinical applicability point of view, this is a desired parameter. For covalent conjugation, a disulfide bridge formation between the CPP and cargo has been used most often, as this linkage is conveniently cleaved in the reductive environment of the cytoplasm. However, covalent conjugation strategy also has its drawbacks. For example, it is very cumbersome to generate conjugates with charged ONs; it is not compatible with the delivery of large nucleic acids, such as pDNA, it seems that covalent conjugation is also not compatible with siRNA delivery; and the synthesis and purification is very laborious and costly. Accordingly, non-covalent approach has gained momentum lately.

Non-covalent strategy for vectorization of ONs with CPPs was first introduced in 1997 by the group of Heitz and Divita with the MPG peptide [31]. Since then this approach has been extended to a variety of bioactive cargos, for example pDNA, peptides, proteins, siRNAs, and SCOs. The strategy relies on electrostatic interactions, where positive charge of the CPPs enables neutralization of the negative charge of the ONs, facilitating condensation of ONs into nanoparticles. Additionally, hydrophobic properties and interactions have also been shown to be involved in the formation and stabilization of the nanoparticles. Compared to covalent strategy, non-covalent approach has been less frequently utilized and the reason for this is probably that most of the CPPs do not enable efficient condensation and/or subsequent delivery of the ON-based cargo, and the latter is mainly a consequence of the endosomal entrapment. Nevertheless, advances have been made recently by using both of these strategies with CPPs for the delivery of nucleic acid.

2.1 CPP-mediated delivery of plasmid DNA (pDNA)

A classical gene therapy is based on the concept that a malfunctioning gene is replaced with functional one by introducing it to the nucleus of cells and expressing it at the levels that would enable the amelioration of the disease phenotype. Except for the viral delivery approaches, utilization of pDNA as a source of genetic material has been most vigorously used. The bottleneck for pDNA delivery with CPPs has been the relatively poor delivery efficiency [32-34]. The reason for this is mainly originating from the fact that most of the unmodified CPPs do not enable efficient pDNA condensation and, even if the particles are taken up, they are unable to escape from the endosomes, therefore, remaining biologically unavailable. Moreover, even if some CPPs show *in vitro* potential, few have displayed the same activity *in vivo*, not even in the case of local delivery. To our knowledge, there are not many successful reports on the systemic delivery. Additionally, many reports have used CPPs as additives to cationic lipids to enhance their delivery efficiency [32,35] or they have been used as uptake enhancers for other delivery vehicles [24,36]. A selection of examples describing CPP-mediated delivery of pDNA will be discussed below and are presented in **Table 2**.

In early reports, different synthetic peptides were shown to mediate pDNA delivery and enhance transgene expression. For example, Wyman *et al.* showed that KALA peptide is capable of forming complexes with pDNA and mediating its intracellular delivery [37]. It was hypothesized that the delivery efficiency of KALA peptide was dependent on its ability to form α-helical structures at acidic conditions in the endosomes. In 1997, Morris *et al.* published a report that is now considered a proof-of-principle study for non-covalent nanoparticle formation with CPPs, where they showed that MPG peptide formed nanoparticles with shorter DNA fragments and mediated their intracellular delivery [38]. In 2 years time, this platform was extended to include the delivery of pDNA [39], showing that MPG peptide facilitated the delivery of luciferase-encoding plasmid into various cell lines. Additionally, by delivering pDNA carrying a full-length antisense cDNA encoding human cdc25C, the cell cycle was arrested in large population of cells, preventing their entry to mitosis. In 2002, Rittner *et al.* designed novel amphipathic CPPs, namely ppTG1 and ppTG20, and evaluated their delivery properties both *in vitro* and systemically *in vivo* [40]. They showed that these peptides enhanced the delivery of

Table 2. Examples of the utilization of CPPs for pDNA delivery.

CPP	Gene	Biological effect	Ref.
KALA	Luc	Luciferase expression	[37]
MPG	Luc and cdc25C	Luciferase expression and cell cycle arrest	[39]
Stearyl-Arg8	Luc	Luciferase expression	[26,44]
ppTG1 and ppTG20	Luc	Luciferase expression *in vitro* and *in vivo*	[40]
Macro-branched Tat	Luc	Luciferase expression	[33]
C-5H-Tat-5H-C	Luc	Luciferase expression *in vitro* and *in vivo*	[22]
POD	RFP	RFP expression	[41]
PEG-POD	Luc	Luciferase and β-galactosidase expression *in vitro* and *in vivo*	[42]
PEG-POD	GDNF	GDNF expression *in vitro* and retinal degeneration rescue *in vivo*	[43]
Stearyl-$(RxR)_4$	Luc and EGFP	Luciferase and EGFP expression	[46]
Stearyl-TP10	Luc and EGFP	Luciferase and EGFP expression *in vitro* and luciferase expression *in vivo*	[47]
Stearyl-NLS-Arg8	Luc	Luciferase expression	[45]
TMAF	β-Galactosidase, anti-TF shRNA	β-Galactosidase expression *in vitro* and *in vivo*, anti-TF shRNA expression *in vivo* and tumor reduction	[48]

anti-TF shRNA: Short-hairpin RNA targeting tissue factor; EGFP: Enhanced green fluorescent protein; GDNF: Glial cell line-derived neurotrophic factor; Luc: Luciferase; NLS: Nuclear localization signal; PEG: Polyethyleneglycol; POD: Peptide for ocular delivery; RFP: Red fluorescent protein; TMAF: Tat-Mu peptide's fusion protein with anti-HER2 affibody.

luciferase-encoding pDNA in variety of cell lines. Moreover, luciferase expression was measured from lung tissues upon systemic administration. Unfortunately, these effects were associated with significant toxicity, as many animals in the treatment group died of lung embolism [40]. Lately, Liu *et al.* designed various macro-branched Tat peptide analogs and showed that these were able to deliver pDNA into a variety of cell lines, while transfection efficiency was retained in the presence of serum [33]. More recently, Lo *et al.* reported a Tat peptide analog that was modified with histidines and cysteins, generating the C-5H-Tat-5H-C peptide. This peptide, in complex with pDNA, enhanced luciferase expression in a variety of cell lines. Moreover, after direct injections to the brain and spinal cord of mice, increased luciferase gene expression was achieved [22].

In 2008, Johnson *et al.* described a novel peptide-based vector for ocular delivery of pDNA, named peptide for ocular delivery (POD). In this report, they showed that POD efficiently vectorized pDNA *in vitro* [41]. Later on, POD peptide was shown to be inefficient for *in vivo* delivery and for this it was further modified with polyethylene glycol (PEG) [42]. Interestingly, PEG-POD particles with pDNA were capable of inducing reporter gene expression after local intraretinal administration. Moreover, luciferase expression was induced in lungs after systemic administration of the particles. Finally, the applicability PEG-POD platform was extended even further, with the intraretinal delivery of pDNA encoding glial cell line-derived neurotrophic factor (GDNF) and resulted in a rescue of mouse retinal degeneration [43].

Another line of development has utilized fatty acid-modified CPPs for the formation of peptide-pDNA nanoparticles and gene delivery. In 2001, Futaki *et al.* showed that N-terminal stearylation of the octaarginine (Arg8) peptide enabled efficient pDNA condensation and gene delivery into different cell lines, while the delivery efficiency was reported to be in line with lipofection [26,44]. Furthermore, the stearyl-Arg8 peptide was subsequently used for the functionalization of the MEND, which greatly enhanced its transfection efficiency [24]. Wang *et al.* studied the stearylated nuclear localization signal (NLS)-modified Arg8 peptide (stearyl-NLS-Arg8) for pDNA delivery and showed that it mediated luciferase gene expression *in vitro* [45]. Lately, our group extended stearylation to another arginine-rich peptide, $(RxR)_4$, and reported that this stearyl-$(RxR)_4$ peptide, when formed with pDNA, induced significant increase in gene expression in a variety of cell lines, substantially exceeding the efficiency of regular stearyl-oligoarginine [46]. Also, comparison were made with lipofection and while $(RxR)_4$ exhibited lower efficacy, contrary to Lipofectamine 2000, it did not exert any toxic side-effects to cells.

Another successful example of the utilization of stearic acid modification is the TP10 peptide. Recently, our group showed that previously reported delivery properties of stearyl-TP10 for short ONs can be translated for the delivery of pDNA both *in vitro* and *in vivo* [47]. Stearyl-TP10 formed stable nanoparticles with pDNA and mediated efficient gene delivery in a range of cell lines, with transfection efficiency being comparable to lipofection. Thorough studies concluded that stearyl-TP10 fulfilled many criteria of an efficient transfection agent: transfection of whole cell population in a uniform manner, non-toxic nature, insensitivity to serum proteases, delivery of pDNA with different size, and relative independence of cell confluence. These excellent results in cell cultures encouraged the investigators to test this delivery platform *in vivo*. For this, intramuscular and intradermal injections of stearyl-TP10/pDNA nanoparticles (encoding

luciferase) were carried out and it was shown that these treatments induced substantial local gene expression. Moreover, these treatments were not associated with toxicity or immunogenicity *in vivo*, making stearyl-TP10 an interesting vector for *in vivo* delivery of pDNA [47].

An interesting approach utilizing CPP and targeting ligand fusion protein was reported recently for pDNA delivery. In this, Tat peptide was expressed as a fusion protein with a DNA-binding motif and Human Epidermal Growth Factor Receptor 2 (HER2) antibody mimetic affibody, generating a TMAF vector [48]. TMAF was formulated with protamine and pDNA and this combination enhanced β-galactosidase expression in HER2 over-expressing breast-cancer cell line, but also in breast-cancer xenograft model after intratumoral injection. Notably, after using anti-tissue factor short-hairpin RNA (shRNA)-encoding pDNA, targeted silencing of tissue factor and reduction of tumor size was achieved upon intratumoral injection [48].

2.2 CPP-mediated delivery of splice-correction oligonucleotides (SCOs)

Today, a variety of genetic disorders have been associated with mutations that alter the normal splicing patterns. Since early 1990s, it has been shown that by utilizing short antisense ONs, called splice-correcting ONs (SCOs, also termed splice-switching ONs), it is possible to correct the aberrant splicing patterns and restore the expression of a functional protein [49]. CPPs have been studied extensively for their ability to deliver SCOs. Promising results have been achieved when SCOs, based on peptide nucleic acids (PNAs) and phosporodiamidate morpholino ONs (PMOs), have been covalently conjugated to the CPP, and some of these conjugates have even been taken into the pre-clinical studies [50]. A selection of recent examples describing CPP-mediated delivery of SCOs will be presented below and in **Table 3**.

Early reports showed that covalent PNA conjugates with classical CPPs, such as Tat, penetratin or MAP [51-53], induced significant splicing correction when used at very high conjugate concentrations. Unfortunately, these effects were accompanied with high membrane perturbation and toxicity, which allowed direct penetration across plasma membrane. Consequently, it became evident that these CPPs are not potent enough to be utilized in such context.

Later, many groups intensely studied the possibilities to improve the delivery properties of CPP for the delivery of SCOs. EL Andaloussi *et al.* showed that transportan and M918 peptide, covalently conjugated to PNA, improved SCO delivery as compared to abovementioned classical CPP/PNA conjugates. Even so, high concentrations of conjugates were required to induce significant splicing correction [54,55]. Next, a potent peptide-based vehicle $(RxR)_4$ was reported, where polyarginine peptide was modified with 6-aminohexanoic acid linkers (corresponding to x) [56]. Covalent conjugates of $(RxR)_4$ peptide and PMO ($(RxR)_4$-PMO) induced significant splicing correction *in vitro*, with EC_{50} values being in the low micromolar range [56-59]. $(RxR)_4$-PMO conjugates were also successfully used for *in vivo* delivery of SCO in DMD mouse model, where they were shown to induce functional rescue of the dystrophin protein [57,60,61]. Moreover, AVI Biopharma took these conjugates into pre-clinical studies, targeting severe viral infections and DMD [57,62]. Another recently reported peptide, R6-Pen, which is a penetratin analog elongated with six arginine residues, conjugated with PNA, was shown to reach similar splicing correction efficiency levels than that of $(RxR)_4$-PMO conjugates [63]. Furthermore, the elements from both $(RxR)_4$ and R6-Pen peptides were combined to develop PNA internalization peptides (Pip peptides). When conjugated to PNA, the Pip peptide was shown to be more efficient in inducing splicing correction than previously mentioned vehicles. Notably, the conjugates also induced efficient rescue of dystrophin in DMD model upon local intramuscular administration *in vivo* [64].

Recently there have been significant advances in CPP-mediated delivery of CPP/SCO conjugates, especially in terms of *in vivo* delivery. While the previously mentioned $(RxR)_4$-PMO induced significant splicing correction in DMD model, enabling functional rescue of dystrophin in diaphragm and skeletal muscles, these conjugates were unable to induce efficient dystrophin rescue in the heart tissue [61,65]. Hence, another modified $(RxR)_4$ peptide analog, the B-peptide, was reported. This peptide showed improved delivery properties compared to the parent peptides, as previously unreachable rescue of dystrophin in cardiac muscle was achieved in DMD model [66,67]. Building on that, another vector was created, where the B-peptide was further fused with muscle-specific heptapeptide (MSP). This peptide, conjugated to PMO, showed body-wide dystrophin rescue, leading to the correction of DMD disease phenotype and restoration of muscle function [68,69]. Conclusively, all these reports emphasize the utility of covalent conjugates for splice switching therapy.

Another recent advancement in the field has arisen from the works with non-covalent nanoparticle formation between SCOs and CPPs. The first vectorization of SCOs by non-covalent nanoparticle formation strategy was performed using abovementioned stearyl-TP10 peptide, which efficiently associated with 2′-OMe SCOs and facilitated significant splice correction *in vitro* [28]. Strikingly, stearyl-TP10/SCO nanoparticles induced splice correction at very low SCO concentrations, compared to all previously mentioned CPP/SCO conjugates. Moreover, splice-correction efficiency could be enhanced when the stearic acid was introduced orthogonally to the seventh lysine in the TP10 sequence [70]. Recently, stearyl-TP10 peptide was further modified by adding phosphoryl group to different positions in peptide sequence, resulting in NickFect1 (NF1) and NickFect2 (NF2), and these peptides enabled increased splice-correction efficiency, compared to stearyl-TP10 [71]. Similar N-terminal stearyl-modification was subsequently introduced to the $(RxR)_4$ [46]. Even though stearyl-$(RxR)_4$ did not quit reach

Table 3. Examples of the utilization of CPPs for SCO delivery.

CPP	ON type	Targeted mRNA	Model	Ref.
Tat, Pen	2′-OMe	Luciferase	HeLa pLuc 705	[51]
Tat	PMO	Luciferase	HeLa pLuc 705	[52]
MAP	PNA	Luciferase	HeLa pLuc 705	[53]
Tat, TP, Pen	PNA	Luciferase	HeLa pLuc 705	[54]
M918	PNA	Luciferase	HeLa pLuc 705	[55]
$(RxR)_4$	PMO	Dystrophin	DMD canine myoblasts, DMD mouse model	[57,59-61,65]
Pip peptides	PNA	Luciferase, Dystrophin	HeLa pLuc 705, DMD mouse model	[64]
R6-Pen	PNA	Luciferase	HeLa pLuc 705	[63]
Stearyl-TP10	2′-OMe	Luciferase	HeLa pLuc 705	[28]
NF1	2′-OMe	Luciferase	HeLa pLuc 705	[71]
Stearyl-$(RxR)_4$	2′-OMe	Luciferase	HeLa pLuc 705	[46]
PF14	2′-OMe	Luciferase, dystrophin	HeLa pLuc 705, DMD myotubes	[73]
PF6	2′-OMe	Luciferase	HeLa pLuc 705	[72]
B-peptide	PMO	Dystrophin	DMD mouse model	[66,67]
B-MSP	PMO	Dystrophin	DMD mouse model	[68,69]
P005	PMO	β-Globin	β-Thallasemia mouse model	[100]

2′-OMe: Phospohorothioate 2′-*O*-methyl ONs; DMD: Duchenne muscular dystrophy; HeLa pLuc 705: Model for splice correction efficiency; MAP: Model amphipathic peptide; NF1: NickFect1; PF14: PepFect14; PF6: PepFect6; PMO: Phosphorodiamidate morpholino ONs; PNA: Peptide nucleic acid.

the efficacy levels of stearyl-TP10, 10-fold lower SCO concentration was required to achieve similar effects as with parent $(RxR)_4$-PMO covalent conjugates.

Finally, our group has developed two further modifications of the TP10 peptide, both of which have also successfully been implemented for the delivery of SCO. In the first, the stearyl-TP10 is further conjugated to an endosomotropic entity, yielding a molecule which we designate PF6 (which development is discussed in more detail in the following siRNA delivery section). PF6 mediated a notable increase in splicing correction, exerting similar effects at approximately three times lower ON concentrations than the parent stearyl-TP10 peptide and allowed the correction of the whole transcipt [72]. Interestingly, the increased activity of stearyl-TP10, PF6 and stearyl-$(RxR)_4$ over the parent peptides (TP10 and $(RxR)_4$, respectively) was shown not to be caused by enhanced cellular uptake, but by increased membrane destabilization potential, suggesting that the enhanced endosomal escape underlies their higher biological activity [72]. In the second modification strategy, the lysines in TP10 peptide backbone were substituted with ornithines, with the purpose of increasing stability, particle formation efficiency, and serum stability of the stearyl-TP10 peptide. This vector, designated PepFect14 (PF14), effectively induced splice correction, both in the absence and presence of serum [73]. Notably, PF14 was significantly more efficient than other regularly used delivery vehicles, with the EC_{50} reaching around 100 nM in a reporter cell line, which is significantly lower than any of the most efficient CPP platforms used for the SCO delivery. The feasibility of PF14 was also confirmed in a disease-relevant *in vitro* model of DMD – H2K mdx mouse myotubes. Accordingly, PF14 effectively mediated efficient splice-correction at markedly low concentrations (of around 250 nM), which is a noticeable result, considering that micromolar concentrations can be regarded as a standard level of efficacy for the most efficient peptide-based strategies.

An additional interesting aspect of the same work is that PF14/SCO nanoparticles could be dried and incorporated into solid dispersions [73]. Interestingly, the particles retained their activity after reconstitution, even after being stored at elevated temperatures for several months. To our knowledge, this is the first time when CPP-based nanoparticles have been formulated in this manner and the strategy may have larger impact, as it may be considered for other types of peptide/oligonucleotide systems as well. Therefore, PF14 is an extremely interesting delivery vector for non-covalent vectorization of SCOs.

Conclusively, substantial success in inducing splicing correction with non-covalent CPP/SCO particles has been achieved during recent years. This has resulted in development of more effective delivery systems and decrease in the concentrations of SCOs by nearly one log. Considering that the new approaches offer substantial dose reduction and efficacy in the presence of serum, advances in *in vivo* delivery may also be expected in the near future.

2.3 CPP-mediated delivery of small-interfering RNAs (siRNAs)

RNA interference (RNAi) is a probably the most fundamental addition to the repertoire of gene silencing approaches used in gene therapy settings. RNAi is mediated by short double-stranded RNA (siRNAs) which are fully complementary to their target sequence and when associated with the RNA-induced silencing complex (RISC) they hybridize to the target mRNA and induce the gene silencing by degrading it. The high specificity and silencing activity have made RNAi an

Table 4. Examples of the utilization of CPP for siRNA delivery.

CPP	Target mRNA	Effect	Ref.
MPGΔ^{NLS}	Luc/GAPDH	Luciferase and GAPDH downregulation *in vitro*	[31]
MPG8/Chol-MPG-8	Cyclin B1	Inhibition of the cell proliferation *in vitro*/tumor reduction in mice (Chol-MPG-8)	[80]
CADY	GAPDH	GAPDH downregulation	[81]
Stearyl-Arg8	GFP, MAP2b	GFP and MAP2b downregulation in primary cells	[30]
Stearyl-Arg8 (in MEND)	Luc	Luciferase downregulation	[29]
Stearyl-Arg8 (in MEND)	Luc	Luciferase downregulation in mice	[84]
EB1	Luc	Luciferase downregulation	[21]
Chol-Arg9	VEGF	VEGF downregulation and tumor reduction in mice	[78]
RVG-Arg9	GFP, SOD-1, siFvE^{J}	GFP and SOD-1 downregulation in brain. siFvE^{J} downregulation mediated protection against JEV-induced encephalitis in mice	[79]
Tat-DRBD	dGFP, dRFP, Luc, GAPDH, Oct-4	Efficient gene silencing in difficult-to-transfect primary and suspension cells and in mice luciferase model	[82]
Tat-DRBD	EGFR, Akt2	EGFR and Akt2 downregulation induced tumor reduction and increased survival in mice	[83]
PepFect6	Luc, EGFP, HPRT1, Oct-4, GAPDH	Efficient gene silencing in difficult-to-transfect primary and suspension cells and in mice upon systemic administration in two *in vivo* models	[25]

(dE)GFP: (destabilized enhanced) green fluorescent protein; Akt2: RAC-beta serine/threonine-protein kinase; Chol: Cholesteryl moiety; DRBD: Double-stranded RNA-binding domain; EGFR: Epidermal growth factor receptor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HPRT1: Hypoxanthine phosphoribosyltransferase 1; JEV: Japanese encephalitis virus; Luc: Luciferase; MAP2b: Microtubule-associated protein 2b; MEND: Multifunctional envelope-type nanodevice; NLS: Nuclear localization signal; Oct-4: Octamer-binding transcription factor 4; RVG: Rabies virus glycoprotein; siFvE^{J}: Gene encoding the envelope protein of Japanese encephalitis virus; SOD-1: Superoxide dismutase 1; VEGF: Vascular endothelial growth factor.

extremely appealing gene therapy approach that is investigated in numerous clinical trials for different diseases [2]. In parallel, search for efficient delivery vectors has been ongoing ever since it was discovered that synthetic siRNAs were able to induce gene silencing. CPPs have been also intensely investigated to enhance the delivery of siRNAs, both *in vitro* and *in vivo*. Examples of CPP-mediated delivery of siRNAs will be described below and are also presented in **Table 4**.

In 2003, the group of G. Divita reported the first successful attempt to vectorize siRNAs with CPPs. In this, MPGΔ^{NLS} peptide efficiently vectorized siRNA and induced significant knockdown of luciferase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This was followed by many papers which utilized both covalent and non-covalent approach to vectorize siRNA. However, after a while it became evident that some of the results in these reports were misleading, as it was shown that if the CPP/siRNA conjugates were delicately purified they failed to confer any biological activity [74,75]. Therefore, it was believed that, in the successful reports, where conjugates were not purified, probably the excess of CPP formed non-covalent complexes with siRNAs and these mediated the efficient delivery [76,77]. As a result, it is now believed that a single CPP molecule attached to siRNA is not capable of charge neutralization and does not confer its intracellular delivery, and for this, non-covalent nanoparticle formation approach is most likely the only applicable method to vectorize siRNAs with CPPs. Consequently, the non-covalent nanoparticle approach has been intensely studied for siRNA delivery and many excellent studies have been published.

Lately, our group reported an EB1 peptide, a histidine modified penetratin analog, with endosomolytic properties, which was able to vectorize siRNA in a non-covalent manner and induce significant gene silencing in cell cultures [21]. In parallel, Kim *et al.* reported that cholesterol-modified Arg9 was capable of forming nanoparticles with siRNA. By targeting vascular endothelial growth factor (VEGF), these particles were shown to mediate efficient downregulation of VEGF *in vitro*. Moreover, in a tumor xenograft model these particles mediated VEGF knockdown and subsequent tumor reduction *in vivo* upon intratumoral administration [78]. In a very interesting study, Kumar *et al.* reported targeted delivery of siRNA to the central nervous system using Arg9. In this report, rabies virus glycoprotein (RVG) peptide, a ligand for acethylcholine receptor, was additionally modified with Arg9 to enable siRNA condensation and intracellular delivery. The RVG-Arg9 facilitated efficient nanoparticle formation with siRNA and these particles enabled efficient gene silencing *in vitro*. More importantly, systemic administration of these particles resulted in protection against the fatal viral encephalitis in a mouse model [79]. In another report, MPG-8 peptide mediated efficient siRNA delivery and, when targeting cell cycle regulator cyclin B1, resulted in a sequence-specific gene silencing and decrease in cell proliferation *in vitro*. In addition, these nanoparticles induced significant tumor reduction in a tumor xenograft mouse model upon intratumoral administration. Moreover, if MPG-8 was further modified with cholesterol it mediated efficient tumor reduction and increased the survival of

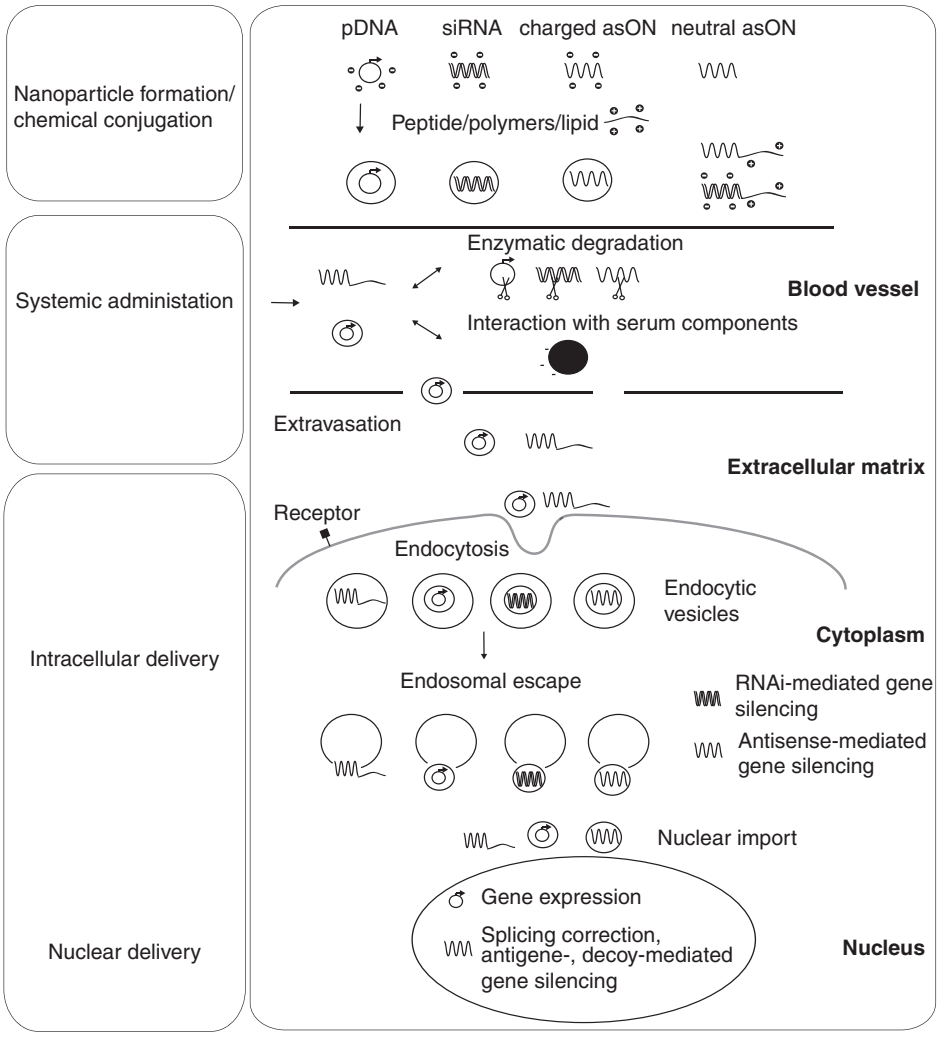

Figure 1. Vectorization of nucleic acids by non-viral vectors and delivery barriers. Non-viral delivery vehicles can vectorize nucleic acids by two routes, either after covalent chemical conjugation between the delivery vector (peptide, polymer or lipid) and nucleic acids or by nanoparticle formation between these entities. It is crucial that these conjugates/nanoparticles are with suitable physicochemical properties and allow the protection of the nucleic acid form the rapid degradation, as after systemic administration, nucleic acids incorporated into nanoparticles/conjugates, are rapidly targeted by different nucleases. Also, unspecific interaction with serum components leads to detection by immune system and unspecific clearance from the body. Moreover, if the size of the conjugates is not large enough they are usually targets for rapid first-pass renal clearance. All of these barriers significantly decrease the half-life of the nanoparticles/conjugates in systemic circulation. Modifying delivery vehicles with PEGs has been shown to improve the properties of delivery vehicles in order to decrease these unwanted interactions. Next, nanoparticles/conjugates have to reach out of the blood vessels and migrate over the extracellular matrix to target tissues and cells. After reaching their target cells, these entities are usually internalized by different types of endocytosis. Consequently, nanoparticles/conjugates get entrapped in the endosomal compartments and this serves as one of the main intracellular bottlenecks for efficient delivery. In order to reach their site of action, delivery vehicle with nucleic acid payload has to escape from the endosomes. Depending on the site-of-action for the nucleic acids, siRNAs and classical asONs, have to escape the endosomes very rapidly as they need to reach cytoplasm. On the contrary, for the molecules that exert their effects in the nucleus, such as pDNA and SCOs, it is more beneficial if they escape endosomes later in the endo/lysosomal pathway. Endosomal escape can be induced by modifying delivery vehicles with different endosomolytic compounds. Importantly, both the cellular uptake and specificity to certain tissues and cells could be increased by the introduction of different targeting ligands to delivery vectors. For cargo that is subject to nuclear delivery, nuclear envelope represents another additional barrier. To some extent utilization of natural NLS sequences has been shown to enable the improvement of nuclear delivery.

the animals upon systemic administration [80]. Subsequently, the group of G. Divita reported a new amphipathic CPP-based delivery vehicle for siRNAs, named CADY. CADY peptide was shown to efficiently form nanoparticles with siRNA targeting GAPDH mRNA and mediated efficient gene silencing in a variety of refractory primary and suspension cells [81].

More recently, the laboratory of S. Dowdy introduced a very interesting CPP-based platform for the delivery of siRNA [82]. In this system, CPP motifs of Tat peptide are expressed as a fusion protein with double-stranded RNA-binding domain (Tat-DRBD). This system was shown to mediate efficient gene knockdown in various hard-to-transfect cell lines, such as human umbilical vein endothelial cells (HUVECs) and human embryonic stem (hES) cells, without exerting any cytotoxic or immunological side-effects. In addition, Tat-DRBD/siRNA complexes were able to mediate efficient luciferase gene silencing *in vivo* after local intranasal administration [82]. Subsequent report showed that Tat-DRBD enabled tumor reduction in a tumor xenograft model upon intratumoral delivery in mice [83]. Unfortunately, no systemic *in vivo* delivery has been reported with this platform.

Stearylation of CPPs has been shown to improve the delivery properties of CPPs for siRNAs. Tönges *et al.* showed that stearyl-Arg8 mediated the delivery of siRNAs to the primary cells, however, the silencing of the expression of EGFP was relatively inefficient [30]. Stearyl-Arg8 has also been used in the MEND platform for surface functionalization to enhance the uptake of these particles. Generally, the core ONs in the MEND system are usually condensed with polylysines or protamines, however, in case of siRNA delivery, these formulations were biologically inactive. Consequently, stearyl-Arg8 was additionally used for the core condensation of siRNAs and these MEND/siRNA particles were shown to mediate silencing luciferase gene both *in vitro* [29] and *in vivo* [84]. Moreover, in these studies it was also noted that stearyl-Arg8 itself, in complex with siRNA, and without being formulated with other components of MEND, did not confer biologically relevant gene silencing.

We have tested a battery of CPP-based vectors for the delivery of siRNA, including the ones described in current review, however, they have all failed to confer reasonable RNAi responses when being non-covalently complexed with siRNAs (unpublished observations). Our results indicate that the reason for this is endosomal sequestration of the complexes. To overcome this limitation we designed a novel endosomotropic modification – a trifluoromethylquinoline moiety – and conjugated four of these molecules to the TP10 peptide. The resultant peptide exerted high efficacy for siRNA delivery *in vitro*, however, only in media devoid of serum proteins. Bearing in mind our previous results that a stearyl moiety increases serum stability of TP10, we further modified this peptide N-terminally with a stearic acid moiety and this resulted in the novel PepFect6 (PF6) peptide [25]. PF6 efficiently condensed siRNA into stable nanoparticles and mediated efficient gene silencing in a range of cell lines *in vitro*, including in various hard-to-transfect cell lines like HUVEC and mouse embryonic stem (mES) cells, without exerting any cytotoxic or immunogenic side effects, while retaining its activity also in the presence of serum. Remarkably, PF6 was able to induce gene knockdown after systemic administration, inhibiting the expression of an endogenous gene in a variety of organs, including liver, kidney and lung. It is notable that while there are several siRNA delivery platforms that are able to induce gene silencing in liver, targeting other organs is only very seldom achieved. All this makes PF6 a very intriguing vector to be used for systemic delivery of siRNAs [25].

Conclusively, there have been significant recent advances in CPP-mediated delivery of siRNAs. However, there are still many limiting obstacles to overcome even in *in vitro* settings. The concentrations of siRNA that is required to induce significant gene silencing with most potent vectors is relatively high and most of these delivery vehicles are very susceptible to the presence of serum. While the number of *in vivo* reports that describe siRNA delivery and gene silencing is increasing, the majority of those have mainly utilized local delivery into tumors and less intriguing information is available for efficient systemic delivery. Therefore, if keeping in mind the potential applicability in clinical settings, there is a significant room for improvements in CPP-mediated siRNA delivery.

3. Conclusions

CPPs have been successfully utilized for the delivery of nucleic acids since their discovery in 1994 [85]. These peptides are characterized by their ability to transfect whole cell populations in a non-toxic manner and mediate the intracellular delivery of genetic material both *in vitro* and *in vivo*. Similarly to other non-viral delivery vehicles, delivery to cell cultures has been often been achieved with reasonable efficiency, however, targeting hard-to-transfect primary cell cultures and delivery *in vivo*, especially systemically, has been a significant obstacle for CPPs. Recently, advances have been made and many novel CPP-based vectors have now also been shown to be compatible with efficient systemic delivery of nucleic acids. One reason for this has been that the emergence of chemically modified CPP-based vectors that can be utilized by the nanoparticle formation approach, as they allow higher efficacy at lower nucleic acids doses. Also, potential of CPPs as uptake enhancers has been lately appreciated and consequently CPPs have been vigorously used as functionalizing units in many other non-viral delivery vehicles, like lipoplexes or polyplexes. Taken together, recent advances in *in vivo* delivery have also meant that CPPs have been successfully used in different *in vivo* disease models, showing that CPPs comprise necessary delivery potential to be applicable in clinical contexts in the future.

4. Expert opinion

Extensive studies with a variety of non-viral vectors have increased the understanding of what is required from an ideal delivery vector. To design delivery vehicles, the following aspects have to be considered (see **Figure 1**). First of all, there are a variety of physicochemical properties, like binding and condensing of the nucleic acids into particles with suitable size, shape, surface charge, and stability in solution. Secondly, if these particles are a subject to the *in vivo* delivery then there different barriers that have to be considered, such as stability and survival in the blood stream, penetration of the blood vessel wall and surrounding tissue and ideally binding to the specific cells. Thirdly, several important general *in vitro* characteristics play role in intracellular delivery, such as stability in the transfection media, including in the presence of serum, association with membrane, and cellular internalization, endosomal escape, cytoplasmic trafficking, nuclear internalization, if necessary, dissociation of the nanoparticles before or at the site of action of the cargo, either in the cytoplasm or nucleus (for extensive reviews see Refs [5,86,87]). Generally, many of these required properties are still not easily controlled, especially simultaneously, and hence, development of nanoparticles with ideal properties is something beyond scientific knowledge and capacity at the moment.

Until now CPP field has mostly used single-component CPP vectors that enable vectorization of the nucleic acids after covalent conjugation of cargoes to CPPs [8] or by spontaneous formation of nanoparticles with the nucleic acids [88]. In many cases, these vectors are compatible with *in vivo* delivery of nucleic acids. Nevertheless, while local delivery of different nucleic acids has been achieved, most of the CPPs do not work after systemic administration. In case of CPP/nucleic acid covalent conjugates, they are usually small enough to undergo first passage clearance through renal system and are still easily accessible to the degrading enzymes after systemic administration. As a result, their serum half-life is too short to mediate efficient delivery. One possible solution to overcome this is to graft the CPP–nucleic acid conjugates to larger delivery platforms, such as dendrimers [89].

Nanoparticle-based systems can have their pros and cons but for systemic delivery they have also many inherent advantages. For example, the size of the nanoparticles reduces their clearance by the kidneys and condensation into the nanoparticle offers better protection to nucleic acids from the degradation [90]. Moreover, suitable size range enables passive targeting of tumors by enhanced permeability-retention (EPR) effect [91]. Nevertheless, nanoparticles are often well detected by the reticuloendothelial system (RES) and that increases nonspecific clearance from the body. Also, nanoparticles often tend to have stability issues and many stimuli could trigger the aggregation of particles and therefore lead to increased clearance or, more alarmingly, induce toxic side-effects (embolism in the lungs). This could be overcome by introduction of hydrophilic polymers, for instance polyethylene glycols (PEGs, a process usually called PEGylation), that shield the nanoparticles from the unwanted interactions and also have positive effects on the physicochemical properties of nanoparticles, all of which contribute to the extended half-life in the blood stream [92]. In case of CPP-based nanoparticles, PEGylation has not gained as much attention that it should, apart from some of the rare examples [42].

Similarly to many other non-viral delivery platforms, CPP share similar inherent weakness – the delivery agent does not distinguish between different cells and tissues. This could be overcome by addition of different targeting ligands; and in the context of non-viral delivery this has been utilized for decades [93]. Nevertheless, as mentioned above, CPPs have been mostly used as single-component systems and there are not many examples of CPPs being functionalized with targeting ligands [79]. Most commonly rather intracellular targeting to the nucleus by nuclear localization signal (NLS) peptide sequences have been utilized [38]. Therefore, this is a relatively unexplored area in the field of CPPs and something that definitely should be pursued intensely.

In the functionalizing CPPs, most work has been conducted in the introduction of different units that would enable endosomal escape [18]. One way has been designing peptide amino acid sequences that have inherent membrane-active or endosomolytic activity. For example, CPPs with secondary amphipathic structure have been shown to be more active, introduction of histidines has been shown render the CPPs more protonatable, peptide sequences with endosomolytic/membranolytic viral-origin have been introduced to the peptide sequences, in order to confer the endosomolytic activity. Recently, also chemical modification has been shown to increase the endosomal escape [94]. For example different hydrophobic modifications, for instance addition of different fatty acids, have been shown to at least partially enable endosomal escape, while introduction of different protonatable modifications has also been shown to have substantial effect on the endosomal escape and subsequently on the bioavailability of CPPs.

Taken together, functionalization of CPP-based vectors is one of the fundamental requirements in the future, if CPPs are to be used for systemic delivery and in clinically relevant settings. Most significantly, methodologies that would allow CPP vectors to be compatible with systemic delivery, delivery to the specific tissues and cells, bioresponsible for endosomal escape, enable efficient intracellular delivery and, above all, how to introduce different modifications simultaneously should be most sought for. While CPP are generally regarded as relatively non-toxic delivery vectors, thorough long-term toxicity evaluations *in vivo* are mostly still lacking and future studies should assign this. An additional aspect that has to be addressed is that until now too much work

is conducted in irrelevant artificial reporter systems and clinically relevant disease models have received less attention. Therefore, in order to understand the true delivery and therapeutic potential of CPP-based systems it is of utmost importance to conduct the future experiments with one eye on instant application in disease models, pre-clinical and clinical applications.

Declaration of interest

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Affiliation

Taavi Lehto[†1], Kaido Kurrikoff[1] & Ülo Langel[1,2]
[†]Author for correspondence
[1]University of Tartu,
Institute of Technology,
Laboratory of Molecular Biotechnology,
Nooruse 1, 50411 Tartu, Estonia
Tel: +372 7 37 4866;
Fax: +372 7 37 4900;
E-mail: tlehto@ut.ee
[2]Stockholm University,
Department of Neurochemistry,
The Arrhenius Laboratories for Natural Sciences,
SE-10691 Stockholm, Sweden